

ENTERIC INFECTIONS IN YOUNG RUMINANTS:
STUDIES ON ESCHERICHIA COLI AND
CRYPTOSPORIDIUM SP

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DECLARATION

The work reported in this thesis was part of a larger project concerned with investigations on neonatal calf diarrhoea, and consequently some of the experiments were carried out in collaboration with my colleagues at the Moredun Research Institute. The work presented in this thesis was carried out by myself, except for some areas of collaborative experiments which are clearly indicated. A full role was played in the design of the multi-discipline experiments and the interpretation of the results.

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UNIVERSITY OF EDINBURGH

ABSTRACT OF THESIS (Regulation 7.9)

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Title of Thesis Enteric Infections in Young Ruminants: Studies on *Escherichia coli* and *Cryptosporidium* sp.

The primary aim of this study was to investigate the prevalence and pathogenic characteristics of enterotoxigenic *E. coli* (ETEC) in diarrhoeic calves in Britain. Developing from the results obtained were studies on a combined vaccine for passive protection of neonatal calves against ETEC and rotavirus infections, *Cryptosporidium* sp infections in laboratory mice, calves and lambs, and the effects of mixed enteric infections in calves and lambs using ETEC, *Cryptosporidium* sp and rotavirus.

Eighty-eight of 1529 *E. coli* isolates (5.75%) from diarrhoeic and clinically normal calves in Scotland and Northern England were found to possess the K99 antigen (K99⁺). There was complete correlation between possession of K99, heat stable enterotoxin (St_a) production and activity in the calf ligated intestinal loop test. All K99⁺ ETEC were isolated from 23 of 306 (7.5%) diarrhoeic calves (1 to 3 days of age) and from 8 of 70 (11.4%) diarrhoea outbreaks. A survey of bovine sera for K99 antibodies by ELISA found 2.96% and 3.9% of sera from calves and cows respectively to be sero-positive.

An ELISA specific for the detection of K99 antigen was used to evaluate the serological response of laboratory animals and cows vaccinated with a K99/rotavirus vaccine. Cows vaccinated with an oil adjuvant vaccine comprising a K99 antigen extract from ETEC strain B41 (O101:K-:K99) and inactivated calf rotavirus, induced high K99 antibody responses in sera and colostrum, with prolonged antibody secretion in milk for 7 to 14 days post parturition. Calves from vaccinated dams were protected against experimentally induced enterotoxigenic colibacillosis by challenge with ETEC strain B44 (O9:K30:K99). The vaccine also significantly reduced faecal excretion of the challenge ETEC in calves from vaccinated dams.

Calf faeces containing *Cryptosporidium* sp were used to subclinically infect 8 strains of laboratory mice at 1 to 4 days of age; transient infections only could be established in 21-day-old mice. Laboratory storage conditions for *Cryptosporidium* sp were investigated, freezing inactivated *Cryptosporidium* sp, but storage was possible at 4°C in PBS or 2.5% potassium dichromate for 4 to 6 months. The parasite could be repeatedly passaged in mice without loss of pathogenicity for gnotobiotic lambs and a system using suckling mice was used to quantify the infectivity of experimental inocula.

Clinical and subclinical infections were established using *Cryptosporidium* sp in 5 to 10-day-old SPF and conventional calves. Pathological changes associated with infection by the organism were found predominantly in the distal small intestine.

Mixed enteric infections were studied in gnotobiotic lambs and conventional calves. Clinical infections were established by inoculation of gnotobiotic lambs with either ETEC, *Cryptosporidium* sp or lamb rotavirus at less than 2 days of age. At 4 days of age or older only subclinical infections could be established using either ETEC, rotavirus or ETEC and rotavirus. Clinical infections were induced with *Cryptosporidium* sp either on its own or in conjunction with ETEC or rotavirus in gnotobiotic lambs 6 days of age or older. There was no evidence from these experiments to suggest that *Cryptosporidium* sp or lamb rotavirus enhanced the pathogenic effect of ETEC in 4 to 7-day-old gnotobiotic lambs.

Only subclinical infections were induced in 9-day-old conventional calves inoculated with ETEC; *Cryptosporidium* sp at this age produced variable clinical responses. Dual infection using ETEC and *Cryptosporidium* sp could not be analysed because of the inadequacy of data.

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ABBREVIATIONS

| | |
|--------------------|--|
| BSA | bovine serum albumin |
| cfu | colony forming units |
| CYE | casamino acids yeast extract broth |
| E'59 | Eagles-59 medium |
| ELISA | enzyme-linked immunosorbent assay |
| FITC | fluorescein isothiocyanate |
| HAI | haemagglutination inhibition assay |
| HE | haematoxylin and eosin |
| HEHA | haemadsorption-elution-haemagglutination assay |
| IF | indirect immunofluorescence |
| IM | intramuscular |
| i.p. | intraperitoneal |
| I.V.. | intravenous |
| LT | labile toxin |
| MID ₅₀ | 50% mouse infective dose |
| NB | nutrient broth |
| OD | optical density |
| PBS | phosphate buffered saline |
| PHA | passive haemagglutination assay |
| p.i. | post inoculation |
| PW | peptone water |
| SC | subcutaneous |
| SPF | specific pathogen free |
| SSB | sodium selenite broth |
| STa | stable toxin |
| TCID ₅₀ | 50% tissue culture infective dose |
| TSB | trypticase soy broth |
| v | volume |
| V.I.C. | Veterinary Investigation Centre |
| VT | Vero cytotoxin |
| w | weight |

CHAPTER 1

REVIEW OF THE LITERATURE

Introduction

Diarrhoea is considered to be one of the most important causes of disease in neonatal animals. Research on neonatal diarrhoea is complicated not only by its multifactorial infectious nature (Tzipori, 1981), but also by environmental, nutritional, immunological and genetic factors. The micro-environment of the gut is complex and affected by both the biochemical and physiological processes associated with digestion and absorption of food and by the presence of gut microflora. Thus, when a micro-organism infects a host many interactions will occur in this complex biological system.

In the past Escherichia coli was believed to be paramount in the aetiology of calf diarrhoea, but since the late 1960's viral agents such as rotavirus and coronavirus have become recognised as enteropathogens, and in the last 5 years the intestinal protozoan, Cryptosporidium sp, has emerged as a possible pathogen. The work described in this thesis concerns the roles of enteropathogenic E. coli and Cryptosporidium sp in neonatal calf diarrhoea.

Human and animal enteropathogenic E. coli have been extensively studied and characterised; some strains invade the intestinal epithelium (Formal, Genski, Gianella and Takeuchi, 1976) but the majority, the non-invasive strains, produce gut-adhesive antigens and elaborate enterotoxins which can be detected by both in vitro and in vivo techniques. These two factors are essential virulence characters for the initiation of diarrhoea and enteropathogenic

E. coli possessing these have been named enterotoxigenic E. coli (ETEC). Aspects of lamb, pig and human ETEC have been reviewed by Sojka (1971); Moon (1974; 1976); Smith (1976); Moon, Isaacson and Pohlenz (1979); Freter (1980) and Beachey (1981). Reference to infection in these species will be largely omitted from this review and emphasis will be placed on ETEC in calves.

Commercial vaccines are available in North America for the prevention of neonatal calf diarrhoea caused by ETEC, rotavirus and coronavirus, but the efficacy of some of these preparations has not been proven. At the present time these vaccines are not licensed for use in the U.K. but their use will be discussed because of their relevance to vaccine development in this country.

Finally, this literature review will mainly consider reports published before the end of 1979; any relevant information published after this time will be considered in the discussion sections following individual chapters.

Surveys on calf mortality in Britain

Early surveys have been reviewed by Lovell (1955) and the Veterinary Investigation Service M.A.F.F. (1964). The most recent calf mortality surveys were conducted in 1962/63 by Leech, Macrae and Menzies (1968) and in 1970 to 1974 by Kilkenny and Rutter (1975).

Jordan (1933) in a survey on mortality in heifer calves reported deaths in 20 to 24% of the calves studied. These results were supported by observations from a similar survey by Smith (1934). A comprehensive survey of dairy calf mortality was undertaken by

Lovell and Bradford-Hill (1940) in England, Wales and Scotland. Of the calves studied 5.5% and 11.4% died before 6 months of age in England/Wales and Scotland respectively. As a result of this work a 3 year survey was undertaken by Withers (1952) on 44 herds (35 dairy and 9 beef herds) in Britain. The average mortality of heifer calves up to 6 months of age was 6.8% in England/Wales and 11.9% in Scotland; 43% of the deaths were attributed to infectious causes, with 12% due to 'E. coli scours'.

Kilkenny and Rutter (1975) calculated a mean calf mortality of 5% during 1970 to 1974 for calves up to 3 months of age in rearing units. Enteric disease and septicaemia due to E. coli and Salmonella spp infections accounted for 70% of the diagnoses during the first 2 months of life, but it was recognised in this survey that the causes of death by enteropathogens may have been incorrectly assessed because of the lack of knowledge on the aetiology and pathogenesis of calf diarrhoea.

Economic loss due to calf diarrhoea

The financial loss to farmers in Britain as a result of diarrhoea in calves is difficult to estimate because of 2 factors. Firstly, the percentage calf mortality due to diarrhoea has not been accurately determined, and secondly, 'hidden' costs due to treatment, extra labour and unthrifty animals have not been assessed.

Kilkenny (1975) estimated an annual loss in potential beef value to be £66 million, the estimate being based on an annual calf mortality of 9.4% for 3.5 million calves. However, the financial losses due to calf diarrhoea were not estimated.

Enterotoxigenic E. coli

(a) The initial recognition of ETEC strains

Smith and Halls (1967a) adapted a test used in the study of cholera (De and Chatterje, 1953) to characterise strains of E. coli isolated from neonatal diarrhoea in a variety of animals and man. This test involved the inoculation of ligated intestinal loops in pigs, calves, lambs and rabbits with the E. coli strains. Of the calf E. coli isolates tested, 7 of 127 induced fluid accumulation in calf ligated loops. All the loop dilating strains caused severe diarrhoea when calves less than 20 hours old were orally inoculated, whereas non-loop dilating strains did not cause diarrhoea. These E. coli were described as enteropathogenic E. coli and the non-loop dilating strains as non-enteropathogenic E. coli. Some of the enteropathogenic isolates only dilated ligated loops prepared in the animal species from which the strain had been isolated.

The dilation of ligated intestinal loops was found to be due to the effects of extracellular toxic substances, called enterotoxins, which were shown either to be heat stable (Smith and Halls, 1967b) or heat labile (Smith and Gyles, 1970). The enteropathogenic E. coli which elaborate either one or both of these enterotoxins were described more specifically as ETEC.

High bacterial counts were present in the contents of the small intestine of diarrhoeic calves orally inoculated with ETEC. Calves infected with pig ETEC did not become diarrhoeic and had low bacterial counts in the small intestine (Smith and Halls, 1967a). Thus it was deduced that there was a virulence factor specific to calf ETEC that enabled the organisms to colonise and proliferate in the intestine of calves.

In comparison to the K88 antigen (Orskov and Orskov, 1966) which was shown to be present on pig ETEC and to mediate adherence to the small intestine of piglets (Jones and Rutter, 1972), calf ETEC were shown to share a surface antigen (Smith and Linggood, 1972) called K99 (Orskov, Orskov, Smith and Sojka, 1975). The K99 antigen was shown to mediate adherence of ETEC to the small intestines of young calves (Isaacson, Moon and Schneider, 1978).

The two major virulence attributes associated with calf ETEC i.e. K99 antigen and enterotoxin production will be described in detail in the following sections.

(b) The K99 antigen

The serological classification of E. coli is based upon analysis of 3 types of antigens, O, K and H (Sojka, 1971). The somatic 'O' antigens are thermostable (121 °C for 2 hours), lipopolysaccharide complexes that form part of the cell wall of these bacteria. 'K' antigens are envelope or capsular antigens of which there are 3 types, L, A and B differentiated by their thermostability. L antigens are destroyed by heat at 100 °C, A antigens are destroyed by autoclaving at 121 °C for 2 hours and B antigens become O-agglutinable by heating to 100 °C. 'H' flagellar antigens are inactivated by heat at 100 °C.

ETEC associated with lamb and calf diarrhoea belong predominantly to the O8, O9, O101 and O20 serogroups with individual strains having different K (A) and H antigens. (Smith and Halls, 1967a; Sojka, 1971; Orskov, Orskov, Smith and Sojka, 1975).

Sojka (1971) was the first to observe that calf and lamb ETEC strains had a closely related K (L) antigen. The antigen, was named 'Kco' by Smith and Linggood (1972) and was found to be present on ETEC able to proliferate in the small intestines of young calves,

while 'Kco' negative isolates could not proliferate. 'Kco' was thought to be comparable to the adhesive antigen present on pig ETEC (Moon, 1974; Rutter, 1975). The antigen was re-named K99 by Orskov, Orskov, Smith and Sojka (1975) and was shown to be the product of the expression of a transmissible plasmid. K99 was thermolabile and not synthesised when the ETEC were grown at 18 °C.

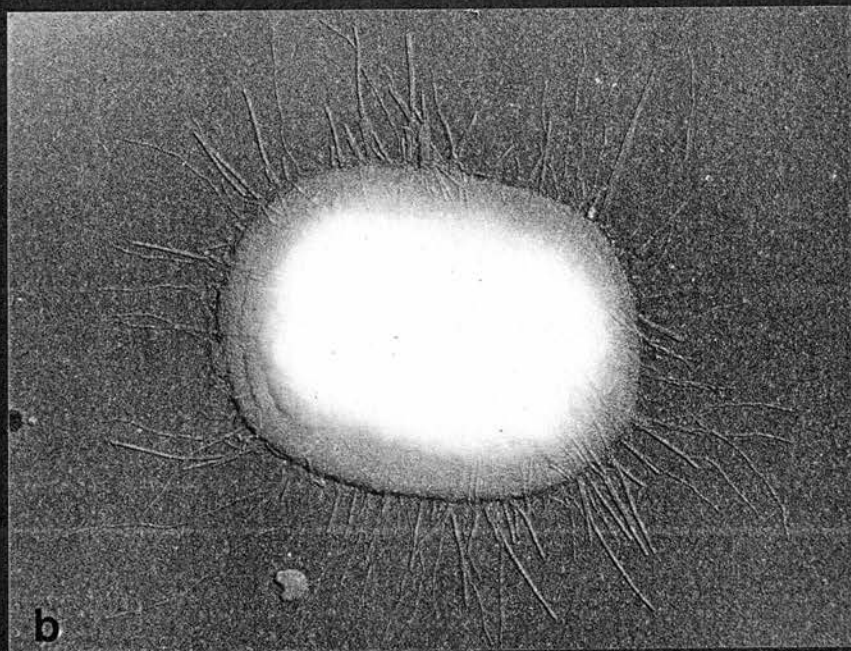
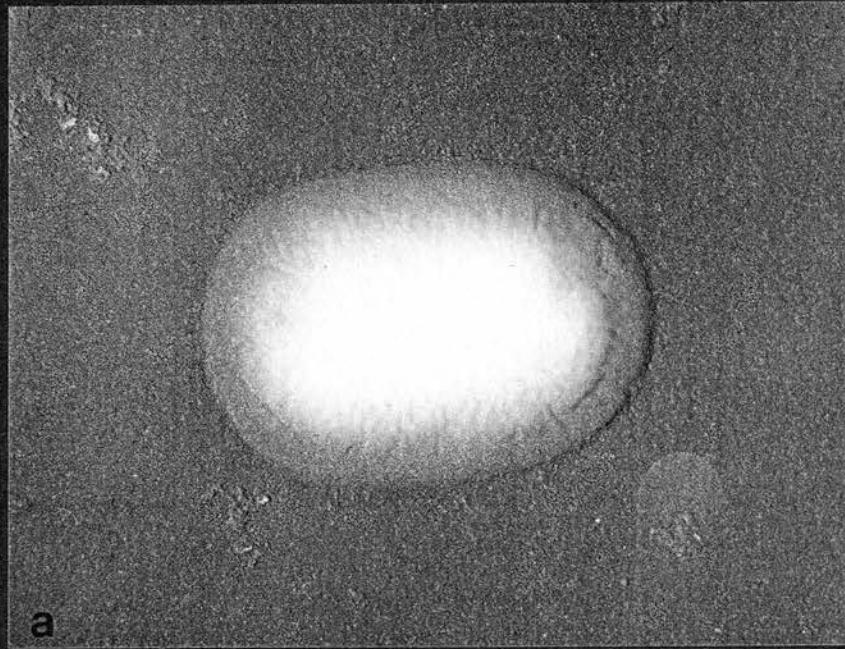
Analysis of purified K99 antigen (Isaacson, 1977; Morris, Stevens and Sojka, 1977) showed that it was mainly composed of protein although traces of carbohydrate were present; periodate oxidation and bacterial protease treatment confirmed the chemical nature of the antigen. Purified K99 was composed of two subunits, the major component having a molecular weight of 22, 500 and a minor component of 29,500. Under immunoelectrophoretic conditions the purified preparation was cationic, had an isoelectric point of 10 and failed to agglutinate guinea pig red cells (Isaacson, 1977). K99 antigen appeared rod-shaped by electron microscopy (8.4nm in diameter and a mean length of 130nm) and was concluded to be a pilus-like structure. Studies by Burrows, Sellwood and Gibbons (1976) showed that K99 antigen caused mannose resistant haemagglutination of sheep erythrocytes, and that the antigen was responsible for the attachment of K99 positive (K99⁺) E. coli to isolated calf intestinal brush borders; cell-free K99 was also shown to inhibit adherence of K99⁺ E. coli to brush borders. Strains B41 (K99⁺) and K12:K99 were shown by electron microscopy to possess filamentous appendages (pili) covering the whole surface of the organisms (Figure 1.1); these pili were absent on K99⁻ E. coli.

Figure 1.1 Gold shadow cast electron micrographs of;

(a) K99⁻ E. coli.

(b) K99⁺ E. coli. Note the radiating fimbriae (pili)
from the surface of the bacterium. (x30,000)

6a



Morris, Stevens and Sojka (1978a) demonstrated that cell free K99 antigen extracted from strain B41 (O101:K99) had an isoelectric point of 4.2, that it was anionic, and that it agglutinated guinea pig and sheep erythrocytes. These results were in contrast to those described previously by Isaacson (1977) in which K99 isolated by ammonium sulphate precipitation was cationic and had an isoelectric point of >10. Further work by Morris, Stevens and Sojka (1978b) clarified this discrepancy by reporting two K99 components, one being anionic, the other cationic. The cationic component was demonstrated on all K99⁺ ETEC serotypes whereas the anionic component was found only in conjunction with the cationic component and only on organisms of the O9 and O101 serogroups (Morris, Thorns and Sojka, 1980). Experiments using the isolated anionic component suggested that it also had adhesive properties.

The serological detection of K99 antigen on ETEC grown in vitro using either tube or slide agglutination was found to be difficult although the problem was partially resolved by the use of TGXA medium (Orskov, Orskov, Smith and Sojka, 1975) or a buffered minimal medium, minca (Guinee, Jansen and Agterberg, 1976). Further modification of the minca medium to include a vitamin supplement, Isovitalex, improved the in vitro detection of K99 on ETEC (Guinee, Veldkamp and Jansen, 1977). It was considered that growth on conventional media enhanced the production of K polysaccharide antigens which 'masked' the K99 antigen making the bacteria non-agglutinable in slide tests using K99 antiserum. A recent report by de Graaf, Klaasen-Boor and Hees (1980) demonstrated that L-alanine suppressed the expression of K99 antigen in vitro and suggested that

the presence of the amino acid in nutrient media was sufficient to have an inhibitory effect on K99 synthesis.

An alternative method was provided by the development of an enzyme-linked-immunosorbent assay (ELISA) (Ellens, de Leeuw and Rozemond, 1979) to detect K99⁺ E. coli in faeces. This method was found to compare favourably in sensitivity with the minca-Isovitalex/slide agglutination method. By a simple modification, the ELISA was adapted to test for antibodies to the K99 antigen in sera and whey (Ellens, de Leeuw and Rozemond, 1979).

The majority of calf ETEC possessed the K99 antigen, but in some cases enterotoxin producing strains were isolated in which K99 antigen could not be detected, e.g. Myers and Guinee (1976), 28 of 35 ETEC were K99⁺; Braaten and Myers (1977) 172 of 177 ETEC were K99⁺; Isaacson, Schneider and Moon (1978), 86% ETEC were K99⁺. The apparent absence of K99 may in most cases be merely due to failure to demonstrate K99 antigen in vitro particularly those analyses carried out before the use of minca-Isovitalex, or it may be possible that these strains carry other adhesive antigens. The 987P antigen originally found on porcine ETEC (Nagy, Moon and Isaacson, 1977) was demonstrated on 5 of 7 K99⁻ calf ETEC isolates (Isaacson, Schneider and Moon, 1978) and to date there is one report of K88⁺ ETEC isolated from calves with diarrhoea (Ellis and Kienholz, 1977). Conversely K99 antigen, originally thought to be the specific adhesive antigen of lamb and calf ETEC, was detected on porcine ETEC (Moon, Nagy, Isaacson and Orskov, 1977) and Smith and Huggins (1978) were able to demonstrate that a calf K99⁺ ETEC could adhere to the small intestine of piglets and cause diarrhoea.

(c) Enterotoxins produced by ETEC

By means of piglet ligated intestinal loops Smith and Halls (1967b) demonstrated an enterotoxin in bacteria free culture media. The enterotoxin retained its activity after heating to 100 °C for 30 minutes, and was called heat stable toxin (ST) by Smith and Gyles (1970). Gyles and Barnum (1969) also using piglet ligated loops demonstrated an apparently different enterotoxin which was elaborated by some pig ETEC. This enterotoxin was inactivated by heat at 60 °C for 30 minutes and called heat labile toxin (LT) (Smith and Gyles, 1970).

Bacterial conjugation experiments demonstrated that the production of both toxins was controlled by transmissible plasmid(s) but only ST/LT recipient strains were isolated when ST/LT donor strains were used.

(i) Heat stable toxin (ST)

ST was shown to be produced by all pig ETEC and appeared to be non-antigenic (Smith and Gyles, 1970). Gyles, So and Falkow (1974) examined Ent plasmids isolated from human ETEC. Plasmids coding for ST/LT were homogeneous in size and were composed of single stranded DNA (molecular weight 6×10^7 daltons), whereas Ent plasmids coding for ST only were heterogeneous in size (2 to 8×10^7 daltons). This suggested that ST may have been heterogeneous in size which was supported by biochemical studies in which the molecular weight of ST was found to be 1,000 to 10,000 daltons (Jacks and Wu, 1974). Kapitany, Scoot, Forsyth, McKenzie and Worthington (1979) suggested that ST of bovine and porcine origin differed significantly with respect to amino acid composition and heat stability.

The biochemical properties of ST have been studied (Jacks and Wu, 1974; Hamilton, Scoot, Roe and Nielson, 1976; Lallier, Lariviere and St-Pierre, 1980; Robertson and Alderette, 1978). The toxin was shown to be acid resistant (pH 2 to 10) and composed of 15% protein and 2% carbohydrate. It was also resistant to pronase, lipase and amylase treatment. Although ST has been considered to be non-antigenic, Lallier, Lariviere and St-Pierre (1980) prepared antiserum to purified porcine ST which neutralised the enterotoxin activity in a cell-free supernate of purified ST.

The infant mouse test was developed by Dean, Ching, Williams and Harden (1972) as an alternative to ligated loops. The original test was shown to be specific for ST, but since its development some limitations have been recognised by Burgess, Bywater, Cowley, Mullan and Newsome (1978) who reported pig ETEC to produce 2 heat stable toxins. The toxins were separated by methanol extraction; STa was methanol soluble, partially heat stable, active in infant mice, active in ligated loops in neonatal piglets and calves but inactive in weaned piglets; STb was only active in ligated loops made in weaned piglets and rabbits. Moon and Whipp (1970) and Gyles (1979) also found 2 forms of ST isolated from pig ETEC. However bovine ETEC have been shown to produce only STa.

The mechanism of action by ST at the molecular level is unclear, but purified ST administered to suckling mice gave a 10-fold increase in basal cyclic guanosine monophosphate (cGMP) (Gianella and Drake, 1979). The cGMP level rose just before fluid secretion into the gut lumen. Studies using homogenates of rabbit and rat intestinal mucosa suggested that stimulation of fluid secretion by ST resulted from

specific activation of guanylate cyclase (Guerrant, Hughes, Chang, Robertson and Munad, 1980). ST may have activity other than the enhancement of fluid secretion. Pesti (1978) demonstrated that ST applied to the mucosal surface of piglet intestine had a depressive effect on the muscularis. This may cause gut stasis which could enhance mucosal attachment of bacteria or even allow a toxin effect without attachment.

(ii) Heat labile toxin (LT)

LT was produced by some pig ETEC (Smith and Gyles, 1970) and human ETEC (Scotland, Gross and Rowe, 1977). In general bovine ETEC did not elaborate this enterotoxin although there were some reports of LT⁺ isolates (Moon, Whipp and Skartvedt, 1976; Ellis and Kienholz, 1977).

Purified LT preparations have exhibited biological properties similar to cholera toxin (Sack, 1980). LT was shown to have a molecular weight of 73,000 and treatment with sodium dodecyl sulphate gave two fragments of molecular weights 44,000 and 30,000 (Kunkel and Robertson, 1979). Unlike ST the biological activity of LT was decreased by trypsin and abolished by pronase, proteinase K and heat at 65 °C for 30 minutes. LT was antigenic and immunologically cross-reactive with cholera toxin as antiserum to cholera toxin neutralised LT activity (Clements and Finkelstein, 1978; Klipstein and Engert, 1977).

Many laboratory tests became available for the detection of LT because of the direct application of research on cholera toxin. The Y1 adrenal mouse tumour cell test was widely used (Donta, Moon and Whipp, 1974) and had been adapted for use in microculture (Sack and Sack, 1975). LT bound to Y1 cells and caused an increase in cyclic

3'-5' adenosine monophosphate (cAMP), which in turn stimulated steroidogenesis. The Y1 cells became rounded with increased steroid production and were easily identified as a marker indicating the presence of LT. Other tissue culture tests for LT included the use of Chinese hamster ovary cells (Guerrant, Brunton, Schaitman, Rebhun and Gilman, 1974) and Vero cells (Speirs, Stavric and Konowalchuk, 1977). ELISA techniques have also been developed exploiting the Gm1 ganglioside binding of LT (Svennerholm and Holmgren, 1978) and its antigenicity (Yolken, Greenberg, Merson, Sack and Kapikian, 1977).

The mode of action of LT on the gut mucosa is very similar to that described for cholera toxin (Sack, 1980). LT binds to Gm1 ganglioside receptors present on the small intestinal epithelium. A series of biochemical events takes place which results in the activation of adenylate cyclase which increases the intracellular concentration of cAMP. The movement of electrolytes into epithelial cells across the plasma membrane of the microvillous border is thought to be controlled by cAMP. Increased cAMP decreases electrolyte entry into the cells and associated water absorption. Back diffusion from intracellular spaces through apical junctional complexes continues resulting in net secretion of electrolytes and water into the intestinal lumen. Active secretion of chloride ions is also stimulated by elevated cAMP levels. Thus the net absorptive flux of fluid and electrolytes which takes place in normal intestine is reversed with resultant luminal accumulation of both fluid and electrolytes.

(d) Enterotoxigenic colibacillosis : Clinical manifestations and pathological changes

ETEC induced diarrhoea was first described by Smith and Halls (1967a). Experimental calves orally dosed at 6 to 20 hours of age with loop dilating E. coli isolates became severely diarrhoeic 9 to 22 hours post inoculation. Large quantities of watery diarrhoea were excreted, with a faecal dry matter as low as 5% (normal faeces 20 to 25% dry matter, Pearson, McNulty and Logan, 1978). A few hours after the onset of diarrhoea the calves became dehydrated, their eyes sunken and the extremities cold. Untreated calves collapsed and died within 8 to 16 hours of the onset of diarrhoea. Experimental calves older than 24 hours of age inoculated with similar calf ETEC isolates did not become diarrhoeic (Smith and Halls, 1967a).

Studies on small intestinal mucosal scrapings demonstrated that calves infected with K99⁺ ETEC had high bacterial counts in the middle and posterior areas of the small intestine. Cryostat sections of the sites stained by the indirect immunofluorescence (IF) test using specific K99 antiserum (Isaacson, Moon and Schneider, 1978) or anti-serum against whole organisms (Pearson, McNulty and Logan, 1978) showed heavy bacterial colonisation of the tips and sides of villi of the middle and posterior small intestine whilst the duodenum remained un-colonised.

Morphological changes have been observed in the intestines of infected calves by histological and electron microscopic studies (Pearson, McNulty and Logan, 1978; Pearson, Logan and Brennan, 1978; Pearson and Logan, 1979; Bellamy and Acres, 1979) even though diarrhoea was probably caused by enterotoxin acting biochemically.

Histological examination indicated stunting and fusion of villi associated with bacterial adherence in the jejunum and ileum. These changes were patchy with some slender villi remaining amongst the majority of stunted structures; affected villi were estimated to be 60% shorter in the ileum of affected calves. The altered villous epithelium was generally cuboidal but some columnar cells were seen. Infiltrates of neutrophils were observed in the lamina propria. Serial killing of infected calves revealed a few bacteria on the distal small intestine as early as 3 hours post inoculation (Pearson and Logan, 1979). After this time adhesion extended anteriorly between 6 and 36 hours with pathological changes becoming evident 6 to 12 hours post inoculation.

Transmission electron microscopic studies of infected intestine (Bellamy and Acres, 1979) indicated that bacteria adhered to the tips of microvilli and were never seen within epithelial cells. Bacteria were separated from microvilli with the presence of 'many thin filamentous structures' in the zone of separation (Bellamy and Acres, 1979). Scanning electron microscopy (Pearson, Logan and Brennan, 1978) has confirmed the histological findings.

Thus, although the pathogenesis of this disease is primarily mediated by the action of an enterotoxin, associated pathological changes occur possibly initiated either by the functional changes or possibly indicating other pathogenic mechanisms.

(e) Prevalence of ETEC

Most of the reported epidemiological surveys of calf diarrhoea have been carried out in Canada and the U.S.A. Initial surveys by Acres, Laing, Saunders and Radostits, (1975) and Morin, Lariviere and Lallier (1976) found ETEC in 11/32 (34%) and 2/55 (3.6%) diarrhoeic

calves respectively. The first survey was conducted on one farm only, with identification of ETEC by ligated loop tests. Smith and Halls (1967a) using the same test identified 7 ETEC from 127 strains obtained from various countries in Europe. ETEC were isolated from 24 of 100 herds studied by Moon, Whipp and Skartvedt (1976). Within these herds 30 of 273 diarrhoeic calves (11%) were infected by ETEC. A study by Myers and Guinee (1976) revealed that ETEC were isolated from calves with diarrhoea in 118 of 355 (33%) herds studied, in total 1,004 E. coli isolates were examined of which 124 were found to be ETEC. A further 2 surveys (Lariviere, Lallier and Morin, 1979; Isaacson, Moon and Schneider, 1978) studied 194 and 32 diarrhoeic calves respectively from which ETEC were isolated in 24 (12%) and 9 calves (28%) respectively. Thus there is a considerable variation in the prevalence of ETEC infections in different surveys.

ETEC have been involved in multiple enteric infections in calves along with rotavirus, coronavirus and Cryptosporidium sp (Morin, Lariviere and Lallier, 1976; Moon, McClurkin, Isaacson, Pohlenz, Skartvedt, Gillette and Baetz, 1978). ETEC infections were commonly detected in calves 1 to 2 days of age in the surveys cited previously, but calves excreting both ETEC and rotavirus tended to be older (2 to 5 days of age) when infection was detected. Experimental evidence showed that severe diarrhoea and death could be induced by infecting colostrum deprived newborn calves with ETEC and rotavirus at doses which would not be lethal if given separately (Gouet, Contrepolis, Dubourguier, Riou, et al, 1978). Thus a synergistic effect may be observed between enteric pathogens during mixed infections.

(f) Passive immunisation of calves against enterotoxigenic colibacillosis

Effective immunisation using ST as an antigen may be difficult because of its weak antigenicity. An alternative approach to the control of this disease would be to inhibit the adherence of ETEC to the small intestine. As calves are susceptible to ETEC infections at a very early age passively acquired antibodies derived from the dam capable of inhibiting adhesion could be essential.

Early experiments on vaccination (Sellers, Smith and Pook, 1962; Gay, MacKay and Barnum, 1964) were unable to protect calves against 'colibacillosis' although neither the disease nor the E. coli vaccine strains were clearly identified. Vaccination using bacterins comprised of the calf ETEC strain B44 (Myers, Newman, Wilson and Catlin, 1973, Newman, Myers, Firehammer and Catlin, 1973) and a formalin killed 6 ETEC strain bacterin (Myers, 1976a) provided protection against challenge with strain B44. Field trials using the 6 strain bacterin (Myers, 1976b) significantly reduced diarrhoea in calves sucking vaccinated mothers.

Myers (1978a) reported that passive protection of calves could also be achieved by vaccination of their dams with various capsular and fimbrial antigen preparations.

Recent studies have used purified K99 antigen extracted from the K12:K99 strain to vaccinate pregnant cows (Acres, Isaacson, Babuik and Kapitan, 1979; Nagy, 1980). Calves from vaccinated mothers were protected from experimental challenge with K99⁺ ETEC strains. It was concluded that K99 antibodies in the colostrum of vaccinated cows protected calves from enterotoxigenic colibacillosis.

Other enteropathogens

(a) Salmonella spp

Salmonellosis in calves tends to spread rapidly and reach epidemic proportions, especially in intensive systems such as calf rearing units. Calves of any age can be infected, but clinical infections occur most commonly at 3 to 6 weeks of age (Richardson, 1975).

Outbreaks of calf salmonellosis are often severe (Richardson and Watson, 1971) and enteritis is a variable feature. As the disease progresses, depression, dehydration and emaciation become evident after the initial onset of fever, anorexia and fetid diarrhoea. Pneumonia, nervous signs and joint swelling may be observed in some animals. Clinically affected calves surviving infection eventually cease to excrete the organism, but adult cattle may become carriers for the rest of their lives. S. typhimurium and particularly S. dublin are the two species predominantly associated with enteric salmonellosis of cattle in England and Wales (Sojka and Field, 1970; Sojka, Wray and Hudson, 1975).

The control of Salmonella spp infections is complicated by the carrier-excretor state in adult cattle, which provide a constant source of infection for the susceptible host. Active immunisation of calves with a live vaccine has been shown to be successful (Smith, 1965) and provides the possibility of controlling infection within an affected herd.

Campylobacter spp

Knowledge concerning the genus Campylobacter has recently been reviewed by Smibert (1978). These organisms which are small, gram-

negative, microaerophilic curved rods cause infections in humans and a variety of animals.

The role of Campylobacter spp in neonatal calf diarrhoea is far from established, but it is known that these organisms may be isolated from diarrhoeic calves (Al-Mashat and Taylor, 1980 a and b).

(c) Rotavirus

Rotaviruses have been extensively reviewed (McNulty, 1978; Flewett and Woode, 1978; Wyatt, Kalica, Mebus, Kim, London et al, 1978) and will be discussed only briefly.

In 1969 Mebus and co-workers demonstrated the presence in calf faeces of a filterable agent which was capable of reproducing diarrhoea in newborn calves. This agent was shown by electron microscopy to be reo-virus-like, 65 to 70nm in diameter with a double layered capsid (Mebus, Underdahl, Rhodes and Twiehaus, 1969). Similar viruses have subsequently been observed in a variety of animals and man (McNulty, 1978) and have been called 'rotavirus' (Flewett, Bryden, Davies, Woode, Bridger and Derrick, 1974). Rotavirus isolates from different animals are morphologically similar, and can be shown to share a common group antigen by a variety of tests.

Rotavirus infections in cattle occur characteristically in young calves, in which clinical symptoms generally include diarrhoea, depression, dehydration and anorexia, with death ensuing in some cases (Woode and Bridger, 1975; McNulty, McFerran, Bryson, Logan and Curran, 1976). The virus multiplies in the intestinal tract, and is excreted exclusively in faeces after a relatively short incubation period of 15 hours to 4 days.

Viral replication was shown by immunofluorescence studies to take place primarily in the epithelial cells on the sides and tips of villi in the small intestine of calves; crypt cells remained uninfected. Infected cells were lost from the tips of villi and were replaced in the normal repair process by accelerated division of immature cells from crypts (Mebus, Stair, Underdahl and Twiehaus, 1971). Pathological changes in the proximal and middle small intestine were associated with areas in which rotavirus antigen was detected by immunofluorescence. The changes included stunting of villi, replacement of columnar cells with cuboidal cells and an infiltration of mononuclear cells, (Mebus, Stair, Underdahl and Twiehaus, 1971; Woode and Bridger, 1975).

Vaccination was attempted using a live attenuated calf rotavirus vaccine administered to calves as soon after birth as possible (Mebus, White, Bass and Twiehaus, 1973). Encouraging results were obtained from initial field trials, but subsequently Acres and Radostits (1976) demonstrated no difference in the incidence and severity of diarrhoea between vaccine and placebo treated calves. Vaccination of the pregnant cow with an inactivated rotavirus vaccine has been considered (Snodgrass, Fahey, Wells, Campbell and Whitelaw, 1980). Calves from vaccinated and placebo treated cows were challenged with rotavirus at 7 days of age. All calves developed diarrhoea, although the onset in calves sucking vaccinated cows was significantly delayed. It was postulated that the delay in onset of diarrhoea indicated a passive immunisation which could have protected calves from a smaller, more realistic natural challenge; the specific antibody bathing the gut lumen was probably overwhelmed by the experimental rotavirus challenge.

(d) Coronavirus

Coronaviruses are a group of lipid containing, pleomorphic, enveloped RNA viruses with distinct surface projections. Coronaviruses cause transmissible gastroenteritis and epidemic diarrhoea in pigs and have been associated in addition with enteric disease in calves, dogs, turkeys and humans (Holmes, 1979).

A coronavirus was first reported in the diarrhoeic faeces of calves by Stair, Rhodes, White and Mebus, (1972), and subsequent challenge studies showed that this calf enteric coronavirus could produce diarrhoea in experimental neonatal calves (Stair, Rhodes, White and Mebus, 1972; Mebus, Newman and Stair, 1975; Bridger, Woode and Meyling, 1978). Immunofluorescence studies demonstrated virus antigen in epithelial cells throughout the small intestine and colon shortly after the onset of diarrhoea, 24 to 48 hours post inoculation. Pathological changes were observed in the small intestine, where villi were stunted and sometimes fused. The pathogenesis of experimental coronavirus and rotavirus infections are similar (Mebus, 1978), but the significance of coronavirus as an enteropathogen remains to be established by field surveys.

(e) Other enteric viruses

Small viruses, in particular, parvoviruses, astroviruses and caliciviruses have been found in diarrhoeic faeces from a variety of animals by electron microscopic examination.

Parvoviruses, which are 22nm in diameter have been detected and experimentally found to cause diarrhoea in calves (Storz, Leary, Carbon and Bates, 1978). Other parvoviruses have been characterised as causes of feline panleucopaenia and acute enteritis and myocarditis

in puppies. These viruses replicate specifically in the nuclei of rapidly dividing cells, in the case of the intestine, in crypt cells. Parvovirus infections are therefore more severe than other enteric viral infections as they destroy cells capable of repopulating the epithelium.

Astrovirus (28nm in diameter) first described by Madely and Cosgrove (1975) in stools from children have been detected in the faeces of diarrhoeic calves (Woode and Bridger, 1978) but experimental infections using this agent did not cause diarrhoea in gnotobiotic calves. In the same study a calici-virus-like agent (33nm in diameter) was shown to be capable of causing diarrhoea in gnotobiotic calves.

The role, significance and occurrence of many of these viruses in neonatal calf diarrhoea still has to be elucidated as they can only be detected by electron microscopy, a facility not available to all diagnostic laboratories.

(f) Cryptosporidium sp

There are conflicting reports as to whether the genus Cryptosporidium consists of many species (Tyzzer, 1907; 1910; 1912; Vetterling, Jarvis, Merrill and Sprinz, 1971; Iseki, 1979) or a single species (Tzipori, Angus, Campbell and Gray, 1980). In this thesis therefore the organism will be referred to by generic name only, coupled with the animal species of origin e.g. calf Cryptosporidium sp.

Taxonomically the genus Cryptosporidium belongs to the order Eucoccidiorida, suborder Eimeriorina and family Cryptosporidiidae. It is a coccidian parasite with various stages in the life-cycle

ranging in size from 3 to 6µm in diameter. Similar organisms have been identified in the gastrointestinal tract of at least 12 species of animals including man (cited by Pohlenz, Bemrick, Moon and Cheville, 1978) and were found attached to the microvillous surface of intestinal epithelial cells. Unlike many other coccidia these organisms have not been observed within the cytoplasm of epithelial cells; whether they are surrounded by a thin membrane of host origin and may therefore be defined as intracellular parasites (Hampton and Rosario, 1966; Vetterling, Takeuchi and Madden, 1971; Kovatch and White, 1972), or whether they are truly extracellular (Tyzzer, 1910; Pohlenz, Bemrick, Moon and Cheville, 1978) is a matter of controversy.

Tyzzer's initial descriptions of Cryptosporidium sp (1907, 1910 and 1912) and its occurrence in the stomach and small intestine of the common mouse remained unconfirmed for over 40 years, until Slavin's work on cryptosporidiosis in turkeys (Slavin, 1955) and the investigations of Hampton and Rosario (1966) in mice. The life cycle of the parasite is at present in dispute. Vetterling, Jervis, Merrill and Sprinz (1971), from their studies in guinea pigs, suggested that a first generation of schizonts gave rise to merozoites which developed into second generation schizonts. The merozoites from the second generation produced gametes for the sexual cycle. No zygote was identified nor was an infectious faecal stage proposed. The life cycle proposed by Iseki (1979) from work on feline Cryptosporidium sp (Figure 1.2) has been supported by studies on human Cryptosporidium sp (Bird and Smith, 1980) although oocysts were not observed in the human work. Iseki claimed that mature oocysts ingested from infected

faeces contained 4 sporozoites which were released to infect intestinal epithelial cells. The endogenous asexual stage passed via the trophozoite (No 2, Figure 1.2) to the developing schizont and finally to the mature schizont, which contained 8 merozoites. Merozoites were released to develop to micro- or macrogametes in the sexual stage. The zygote produced by the sexual cycle matured to an oocyst which was released into the gut lumen. Merozoites may also re-infect the host ('auto-infection') and thence develop by the endogenous asexual stages to produce more merozoites. It was not determined when the asexual and sexual stages occurred during infection.

Cryptosporidium sp have been found associated with cases of calf diarrhoea (Panciera, Thomassen and Garner, 1971; Barker and Carbonell, 1974; Meuten, van Kruiningen and Lein, 1974; Pohlenz, Moon, Cheville and Bemrick, 1978). Calves were commonly infected 4 to 17 days of age, and clinical symptoms included chronic diarrhoea and debilitation. The most prominent pathological changes were found in the ileum, where infected villi appeared blunted and shortened with Cryptosporidium sp attached to epithelial cells. The lamina propria of the ileum was infiltrated with neutrophils, eosinophils and lymphocytes. Histological changes were not always found to be related to the presence of Cryptosporidium sp (Barker and Carbonell, 1974) thus causing a reluctance to consider these changes as specific.

Transmission and scanning electron microscopic studies (Pearson and Logan, 1978a; Pohlenz, Bemrick, Moon and Cheville, 1978) demonstrated Cryptosporidium sp predominantly on the sides and tips of villi. Trophozoites, schizonts, merozoites and gametes were attached

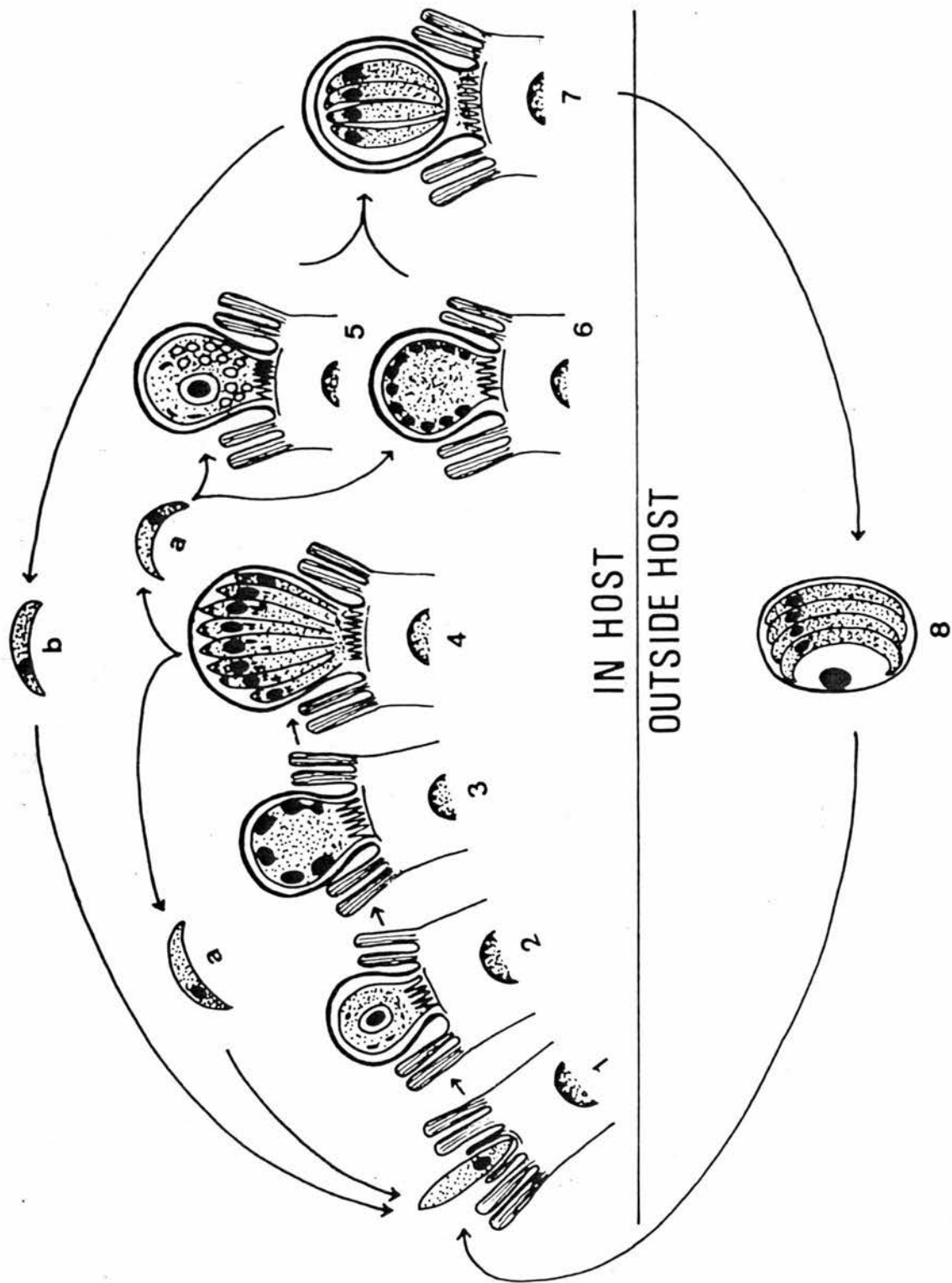
Figure 1.2 Life Cycle of Cryptosporidium sp taken from Iseki (1979)

1 to 4 Asexual cycle of the endogenous stage

1. Sporozoite or merozoite invading the microvillous surface of a small intestinal epithelial cell.
2. A fully grown trophozoite.
3. A developing schizont.
4. A mature schizont with 8 merozoites; a, merozoite released from mature schizont.

5 and 6 Sexual cycle

5. Macrogamete.
6. Microgamete.
7. Mature oocyst containing 4 sporozoites.
8. Oocyst discharged in the faeces; b, sporozoite released from mature oocyst.



to the microvillous surface of epithelial cells, the areas of attachment being devoid of microvilli.

There has been only one report of experimental transmission of Cryptosporidium sp in calves (Pohlenz, Moon, Cheville and Bemrick, 1978). Colostrum deprived calves inoculated with ileal mucosal scrapings from Cryptosporidium sp infected calves developed a profuse watery diarrhoea at 48 hours post inoculation. At necropsy the calves were found to be infected with rotavirus and coronavirus as well as Cryptosporidium sp, so pathological changes could not be specifically ascribed to Cryptosporidium sp infection.

Cryptosporidium sp has been shown to be associated with outbreaks of diarrhoea in calves in North America and Britain (Morin, Lariviere and Lallier, 1976; Moon, McClurkin, Isaacson, Pohlenz, Skartvedt, Gillette and Baetz, 1978; Pohlenz, Moon, Cheville and Bemrick, 1978; Snodgrass, Angus, Gray, Keir and Clerihew, 1980). It remains to be proved experimentally whether Cryptosporidium sp is a pathogen although the histopathological findings of Pohlenz, Bemrick, Moon and Cheville, (1978) suggest that the Cryptosporidium sp infection probably contributed to the pathological changes.

Enteropathogens

The word 'enteropathogens' used throughout this thesis will encompass bacterial, viral and protozoal agents such as ETEC, Salmonella spp, rotavirus, coronavirus and Cryptosporidium sp which have a potential for causing enteric rather than extra-intestinal infection.

Aims of this thesis

The primary consideration of this work was to establish the prevalence of calf ETEC infections in Britain and to examine a K99

antigen/rotavirus vaccine for its ability to passively protect calves against ETEC and rotavirus infections. During the epidemiological survey, Cryptosporidium sp was detected in the faeces of calves, thus work was undertaken to examine experimental Cryptosporidium sp infections in calves and lambs and the effects of mixed enteric infections involving ETEC, Cryptosporidium sp and rotavirus.

CHAPTER 2GENERAL MATERIALS AND METHODS1. MEDIA AND DILUENTS

Media: The following media were used. Each was prepared either according to the manufacturers instructions or as stated.

Casamino Acids Yeast Extract Broth (CYE): this contained casamino acids (Difco) 20g/l, yeast extract (Difco) 6g/l, 0.06M K_2HPO_4 and 1ml of trace salts (0.2M $MgSO_4$, 0.025M $MnCl_2 \cdot 4H_2O$ and 0.031M $FeCl_3$ in distilled water). pH adjusted to 8.5 using 1M NaOH and made up to 1 litre in distilled water.

Dorset egg medium: 12 chicken eggs were homogenised with 100ml of 0.145M NaCl. Slopes were prepared in bijoux bottles by steaming 3ml volumes for 10 to 15 minutes.

Eagles-59 (E'59) medium: Earles basic salts solution, 140ml; lactalbumin hydrolysate, 20ml; 0.1M $NaHCO_3$, 2.5ml; glucose (100g/l), 2ml; vitamins (Flow), 4ml and amino acids without L-glutamine (Gibco), 4ml. Penicillin/Streptomycin solution, 2ml and L-glutamine, 2ml were added just before use.

Earles basic salt solution: 0.018M $CaCl_2 \cdot 2H_2O$, 0.01M $MgSO_4 \cdot 7H_2O$, 0.12M NaCl, 0.05M KCl and 0.01M $NaH_2PO_4 \cdot 2H_2O$, all in distilled water.

L-glutamine: 1.46/l of L-glutamine in distilled water.

Lactalbumin hydrolysate: 50g/l of lactalbumin hydrolysate in Earles basic salt solution.

Penicillin/Streptomycin: 6g/l Penicillin (Glaxo 1700 units/mg) and 10g/l Streptomycin (Glaxo 745 units/mg) in distilled water.

MacConkey agar: (Oxoid Cm 7).

Minca agar: 0.01M KH_2PO_4 ; 0.07M Na_2HPO_4 ; glucose, 1.0g/l; casamino acids, 1.0g/l; trace salts, 1ml (0.041M MgSO_4 , 0.005M $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.001M $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ and 0.003M $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ in distilled water) and 15g/l of agar (Davis).

Minca-Isovitalex agar: Prepared as for minca agar, but the glucose was replaced by 4ml of Isovitalex enrichment (BBL) per litre of medium.

Nutrient broth (NB): (Oxoid Cm 67).

Peptone water (PW): bacteriological peptone (Oxoid L37) and NaCl 5g/l, pH 7.4.

5% sheep blood agar: blood agar base (Oxoid Cm 55) supplemented with 50ml of citrated sheep blood per litre.

Sodium selenite broth (SSB): selenite broth base (Oxoid Cm 395) to which 4g/l of sodium biselenite was added.

Trypticase soy broth (TSB): BBL.

Diluents

5% bovine serum albumin (BSA): 50g/l in distilled water.

Phosphate buffered saline (PBS): 0.14M NaCl, 0.003M KCl, 0.008M Na_2HPO_4 and 0.0015M KH_2PO_4 in distilled water at pH 7.2.

Saline: 0.145M NaCl in distilled water.

Sterilisation: All media and diluents were sterilised by autoclaving at 15lbs steam pressure for 15 minutes except for E'59 and 5% BSA which were millipore filtered (0.45µm average pore diameter).

Suppliers names:

BBL, Cockeysville, Maryland, 421030, USA.

Davis Gelatine Ltd, Leamington Spa, England.

Suppliers names cont'd:

Difco Laboratories, Detroit, Michigan, USA.

Flow Laboratories UK, Irvine, Scotland.

Gibco Europe, Paisley, Scotland.

Oxoid Ltd, Basingstoke, Hampshire, England.

2. E. COLI STRAINS

Table 2.1 summarises the E. coli strains used in laboratory experiments and animal inoculations.

3. BACTERIOLOGICAL METHODS

Isolation of K99⁺ E. coli from faeces

Fresh faecal swabs were plated on 5% sheep blood agar and MacConkey agar. After overnight incubation at 37 °C five representative lactose fermenting colonies were selected and subcultured on minca-Isovitalex agar. After overnight incubation at 37 °C the growth from each colony was tested for K99 antigen. Isolates were stored on Dorset egg medium slopes at 4 °C.

K99 antigen testing by slide agglutination

After growth on minca-Isovitalex agar each of the five isolates were suspended in duplicate drops of saline on a microscope slide. One drop of undiluted anti-K12:K99 adsorbed serum was mixed thoroughly with one preparation. The other suspension acted as a control for auto-agglutination. Bacteria were allowed to react with the antiserum for 10 seconds before the test result was recorded. Any isolate that agglutinated in adsorbed anti-K12:K99 serum was further tested using sera raised to strains EC6, EC46 and B44.

Table 2.1 Origins and characteristics of E. coli strains

| Strain | Source | Species of origin | Serotype |
|---------------|---------------------------------------|-------------------|--|
| EC1 | Colindale, England | Human | 0148:K?:H28, ST ⁺ , LT ⁺ |
| 0159 | " | " | 0159:K?:H34, LT ⁺ |
| B41 | Weybridge, England | Bovine | 0101:K-K99, ST ⁺ |
| B44 | " | " | 09:K30:K99, ST ⁺ |
| Variant B44 | " | " | 09:K30, ST ⁺ |
| " | " | " | 09:K-K99, ST ⁺ |
| " | " | " | 09:K-K99, ST ⁺ |
| " | " | " | 09:K-K99, ST ⁺ |
| EC6 | H. Williams Smith, Houghton, England. | Ovine | 08:K87:K99, ST ⁺ |
| EC7 | " | " | 08:K87, ST ⁺ |
| EC8 | " | " | 08:K85, K99, ST ⁺ |
| EC9 | " | " | 08:K85ab:K99, ST ⁺ |
| H19 | " | " | 026:K?:H?, VT ⁺ |
| K12 | R.E. Isaacson, Iowa, USA. | Human | 07:K12, ST ⁺ |
| (Strain 1475) | " | " | 07:K12:K99, ST ⁺ |
| K12:K99 | " | " | 07:K12:K88, ST ⁺ |
| (Strain 1474) | " | " | 09:K(A):987P, ST ⁺ |
| K12:K88 | " | " | 0101:K?:K99, ST ⁺ |
| (Strain 1476) | " | " | 0101:K-K:F41, ST ⁺ |
| 987 | H.W. Moon, Iowa, USA | Porcine | |
| EC46 | Hoechst, UK, England | " | |
| F41 | N. Chanter, Compton, England | Bovine | |

ST⁺; isolate positive in the infant mouse test for heat stable toxin.
 LT⁺; isolate positive in the Y1 adrenal cell assay for heat labile toxin.
 VT⁺; isolate positive in the Vero cell assay for Vero-cytotoxic factor.
 ?; origin unknown.

Heat stable toxin (STa) test

The assay was modified from the method described by Dean, Ching, Williams and Harden (1972). E. coli isolates were grown overnight in 10ml CYE broth at 37 °C on a rotary shaker. After centrifugation of the broth at 1600g for 20 minutes at 4 °C the supernate was passed through a millipore filter (0.45µm average pore diameter) and stored at -20 °C.

Swiss white mice 1 to 4-days-old were inoculated orally using a 26 gauge needle tipped with teflon tubing. Thawed supernate, coloured by 1% Evans blue dye (w/v in PBS), was administered in 100µl volumes to each mouse; 3 mice were used per supernate. After inoculation mice were kept at 15-18 °C for 4 hours, then killed with chloroform. The intestines from duodenum to rectum were removed and weighed together for each group of 3 mice. The ratio of gut weight to remaining body weight was calculated: ratios less than 0.07 were considered negative; 0.07 to 0.08 doubtful and greater than 0.08 positive for the presence of STa. When doubtful or positive results were obtained a repeat test was performed for confirmation

Labile toxin (LT) test

The Y1 mouse adrenal tumour cell test used was modified from the technique described by Donta and Smith (1974). Bacterial cell-free culture supernates were prepared as described in the STa test, but were tested on the day of preparation or after storage at -70 °C for no longer than 7 days.

100µl of a Y1 adrenal cell suspension, comprising 3×10^5 cells/ml in E'59 medium supplemented with 10% foetal bovine serum and 0.5% $MgCl_2$, was delivered to each well of a microtitre tissue culture

plate (Sterilin M29 ART) and incubated overnight at 37 °C to allow formation of monolayers. Cells were washed once with E'59 medium and finally covered with 50µl of fresh E'59. Test supernate was added to four replicate wells in 50µl volumes, and the plates were incubated at 37 °C for 30 minutes. The overlying fluid was then removed, and 100µl of fresh E'59 medium added. The percentage rounding of Y1 adrenal cells was estimated on unstained monolayers after overnight incubation at 37 °C. The negative control contained CYE broth only, and the positive control a supernate from the growth of strain EC1. Rounding of more than 80% of cells was considered positive. Any result less than this value was repeated.

Vero cytotoxin (VT) test

The method was modified from that of Konowalchuk, Speirs and Stavric (1977). Bacterial cell-free culture supernates were prepared as described for the STa test and were tested on the day of preparation. 100µl of Vero cell suspension at 2×10^5 cells/ml in E'59 medium supplemented with 10% newborn calf serum and 0.5% $MgCl_2$ was delivered to each well of a microtitre tissue culture plate and incubated overnight at 37 °C. 30µl of test supernate was incubated at 37 °C in 4 replicate wells and the cells examined after 24 and 48 hours. CYE broth was used as a negative control and supernate from strain H19 was used as a positive control.

Enumeration of bacteria

The method was modified from that of Miles and Misra (1938). Serial ten-fold dilutions of a bacterial suspension or gut scraping made in PW were dropped in 20µl volumes on to the dry surface of

two 5% sheep blood agar plates, which were then incubated overnight at 37 °C. The titre (colony forming units/ml (cfu/ml)) was calculated from that dilution which yielded 1 to 20 colonies.

Indirect immunofluorescence staining

Cryostat tissue sections on glass slides were placed in a humidified box. Antiserum diluted 1/20 in PBS was applied to the sections and incubated at 37 °C for 30 minutes. Sections were washed in 2 changes of PBS for 15 minutes. Diluted rabbit anti-species immunoglobulin conjugated to fluorescein isothiocyanate (Wellcome Reagents Ltd, Beckenham, England) was applied to the sections at a 1/20 dilution in PBS. Further incubation at 37 °C for 30 minutes was followed by 15 minutes wash in 2 changes of PBS. The cryostat sections were allowed to air dry, then examined for fluorescence in a Leitz Ortholux 2 microscope with a Ploemopak 2 fluorescence vertical illuminator and employing an incident light source from a 50W ultra-high pressure mercury lamp.

Preparation of hyperimmune antiserum

(a) Preparation of OK antisera against K99⁺ *E. coli*

The method was modified from that of Sojka (1968). The K99⁺ *E. coli* strain was cultivated overnight at 37 °C in 10ml TSB, then 0.2ml was spread evenly over a minca-Isovitalex agar plate and incubated overnight at 37 °C. The growth was tested for K99 antigen by slide agglutination using specific anti-K99 serum (kindly donated by Dr. W.H. Jansen Bilthoven, Netherlands). If K99⁺, the growth was harvested in 1/4 strength Ringers solution (0.15M NaCl, 0.006M KCl, and 0.002M CaCl₂ · 2H₂O) and diluted to Browns opacity tube no. 2.

Rabbits were injected intravenously as follows: 0.1ml day 1; 0.2ml day 4; 0.4ml day 7; 0.8ml day 11; and 1.6ml day 14. Fresh inocula were prepared on each occasion and 2 rabbits were used for each strain.

Each rabbit was bled 14 days after the final inoculation. Antiserum was raised against strains B44 (a mean of $10^{9.57}$ cfu/ml injected), EC46 ($10^{9.40}$ /ml), EC6 ($10^{9.51}$ /ml) and K12:K99 ($10^{9.18}$ /ml). The ELISA blocking titres of these antisera are listed in Chapter 4, Section A.

(b) Adsorption of OK antisera

For adsorption a K99 negative (K99⁻) E. coli variant was used. Before use, agglutination in homologous antiserum, but not in specific anti-K99 serum was confirmed. The confluent growth of the strain from 8 minca-Isovitalex agar plates was emulsified in phenol saline (2.5g/l) to make a total volume of 9ml. Antiserum (1ml) raised against the parent K99⁺ strain was added to the suspension which was then incubated at 37 °C for 2 hours. The suspension was centrifuged at 18,000g for 15 minutes at 4 °C then the supernate was passed through a millipore filter (0.45µm average pore size). After overnight dialysis against saline the supernate was stored at -20 °C. The K99⁺ parent strain was agglutinated in the slide test by the undiluted adsorbed antiserum, but no reaction was observed with the K99⁻ variant.

OK sera were also adsorbed on occasions by the homologous E. coli K99⁺ strain grown on 5% sheep blood agar and heat treated at 100 °C for 1 hour.

(c) Preparation of specific anti-K99 serum in a gnotobiotic lamb

(i) Preparation of K99 antigen

E. coli strain B41, grown overnight on 7 minca-Isovitalex agar plates

to provide approximately 10^{11} total cfu, was suspended in 18ml of PBS then vigorously shaken at 60 °C for 30 minutes. The bacteria were pelleted at 20,000g for 30 minutes at 4 °C to obtain supernate 1 and pellet 1.

Supernate 1 was centrifuged at 200,000g for 180 minutes at 4 °C to provide supernate 2 and pellet 2. The ELISA (see Chapter 4) was used to estimate K99 antigen concentration at each preparative stage. (Table 2.2). Pellet 2 was suspended in 1.5ml PBS and dialysed against 0.05M sodium phosphate buffer pH 7.2. The dialysate was loaded on to a pre-equilibrated 4cm x 1cm DEAE - cellulose column (D-52 anion exchanger, Whatman, Maidstone, Kent, England) and developed using 0.05M sodium phosphate buffer pH 7.2. The material bound to the column was eluted with 1M NaCl in 0.05M sodium phosphate buffer pH 7.2. Fractions were collected in 0.5ml volumes and titrated in the ELISA (Figure 2.1). K99 activity was found to be eluted in the void volume as found by Isaacson (1977) although a second peak was eluted with 1M NaCl. Fractions contained in peak 1 (Figure 2.1) were pooled and the protein content estimated by the Lowry method (Lowry, Rosenborough, Farr and Randall, 1951) was found to be 44µg/ml.

(ii) Gnotobiotic lamb inoculation

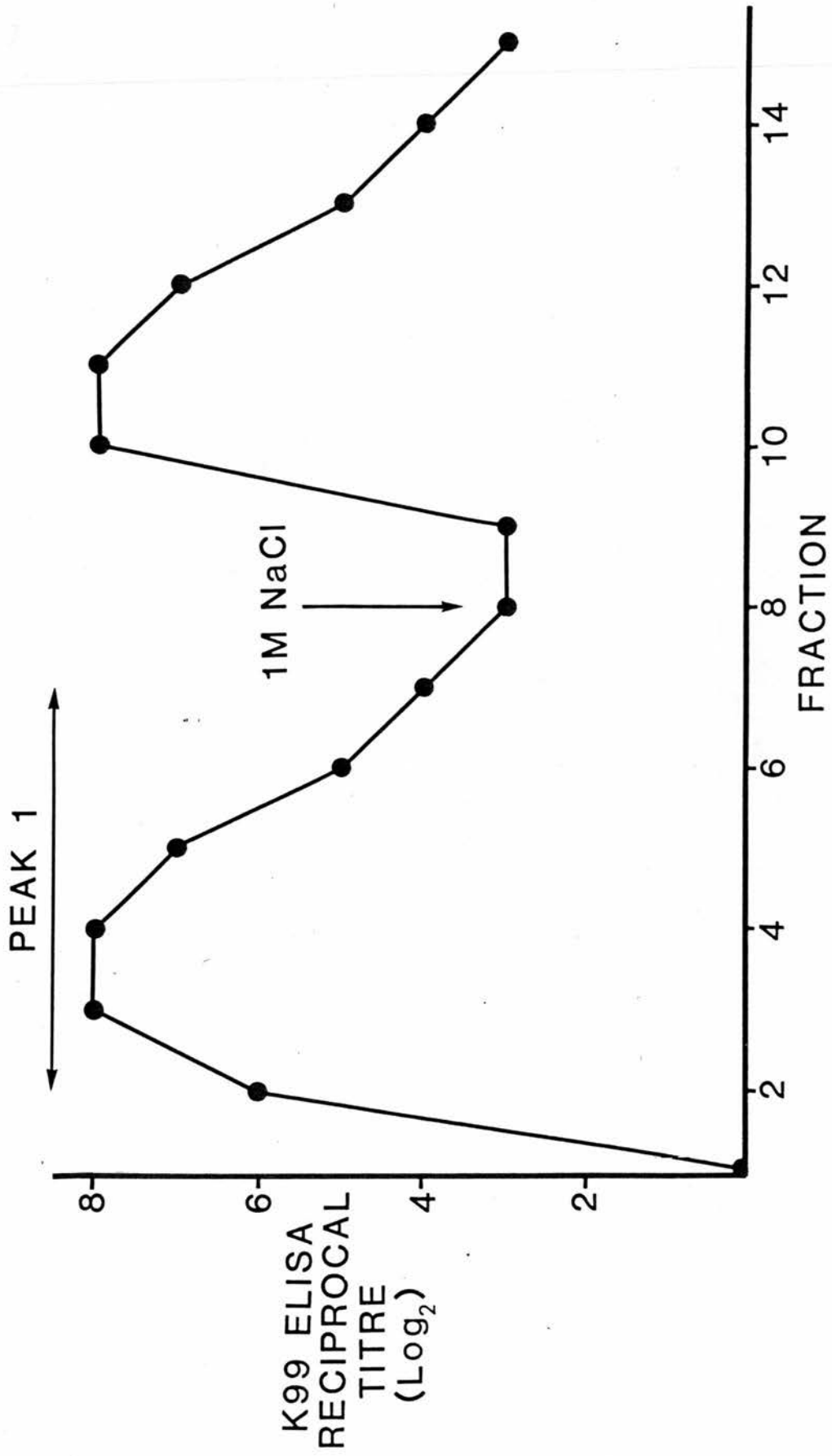
1.2ml of pooled peak 1 was mixed with 60µl of Tween 80 then emulsified with 2.52ml of adjuvant (90% Marcol (Esso) and 10% Arlacel (Sandria Chemicals)). The lamb was inoculated intramuscularly with 3ml of emulsified antigen at 3 separate sites when 5 and 17 days old, and bled at 30 days old. Bacteriological examination revealed the lamb to be bacteria-free until 10 days of age when it became infected

Table 2.2 Titration of supernates and pellets by ELISA for K99 antigen

| Preparation | ELISA end-point titre ^a |
|-------------|------------------------------------|
| Pellet 1 | 80 |
| Supernate 1 | 160 |
| Pellet 2 | 160 |
| Supernate 2 | 20 |

a; Supernates or pellets (suspensions) were serially 2-fold diluted in the ELISA for K99 antigen (see Chapter 4). The reciprocal dilution of test sample that gave an $OD_{405} < 0.1$ was deemed its titre.

Figure 2.1 Fractionation of K99 antigen pellet 2 by DEAE-cellulose column chromatography.



with a haemolytic Staphylococcus sp. The antiserum was found to be specific for the K99 antigen in the slide agglutination test when used against K99⁺ strains at a 1/10 dilution in saline (Table 2.3).

Detection of K99 antigen and antibodies to K99 by immunodiffusion

The method used was similar to that described by Jones and Rutter (1972). Molten agar, comprising 1% Ion agar No. 2 in 0.066M sodium phosphate buffer pH7.0, 0.065M NaN₃, 0.003M disodium ethylene-diamine acetate and 0.145 NaCl, was poured in 4ml volumes onto a 2.5cm x 7.5cm glass microscope slide. Wells, 3mm diameter, were loaded with 10µl of sample (either serum or antigen). Development was allowed for 48 hours at 4 °C in a humidified box.

The agar was dried, stained for 2 minutes by coomassie blue (coomassie brilliant blue, 5g; absolute alcohol, 400ml; glacial acetic acid, 100ml and distilled water 450ml) and cleared in absolute alcohol, 250ml; glacial acetic acid, 100ml and distilled water, 450ml, until background staining was removed. Precipitin lines were stained deep blue and could be observed using a magnifying eye piece.

4. NECROPSY MATERIAL

Material was taken from the following 7 sites in the intestine of calves or lambs under terminal anaesthesia induced by I.V. injection of Sagatal (May and Baker Ltd, Dagenham, England): (1) duodenum, (2) jejunum, (3) mid gut, (4) ileum without Peyers patches, (5) ileum with Peyers patches, (6) spiral colon and (7) caecum. Care was taken to ensure that the blood supply to the gut was maintained during the removal of samples to prevent the development of post mortem artefact as described by Pearson and Logan (1978b).

Table 2.3 Specificity of diluted gnotobiotic
lamb anti-K99 serum in the slide
agglutination test

| Strain | growth temperature ^a on minca-Isovitalex | |
|--------|--|-------|
| | 18 °C | 37 °C |
| EC6 | - | + |
| B44 | - | + |
| B41 | - | + |
| K12 | - | - |
| F41 | - | - |

a; K99 antigen is expressed by K99⁺ strains
when grown at 37 °C but not at 18 °C.

At each of the sites the following samples were obtained from adjacent tissue:

- (a) A 1 to 2cm length of bowel was cut open, laid mucosal side up on a glass petri dish and gently covered with 10% buffered formol saline. Tissue was processed and embedded in paraffin wax, and 5µm thick sections stained with Mayer's haematoxylin and eosin. All histological examinations were carried out by Mr. K.W. Angus, Moredun Research Institute.
- (b) The lumens of 3cm ligated segments were carefully filled with Tissue Tek II (Miles Laboratories, Napperville, Illinois, USA) and the whole segment frozen in a dry ice/isopentane slurry. The frozen material was stored at -70 °C until the preparation of cryostat sections. Sections 6µm thick were attached to glass microscope slides, air dried, and fixed in cold acetone for 10 minutes at 4 °C.
- (c) Segments 1cm long were immersed in 3% glutaraldehyde in sodium phosphate buffer pH 7.4. Tissues were trimmed while immersed in glutaraldehyde into 1mm and 5mm pieces for transmission and scanning electron microscopy respectively. All specimens were post fixed using osmium tetroxide in sodium phosphate buffer pH 7.4. Specimens for transmission electron microscopy were dehydrated in graded alcohols and embedded in araldite. Ultrathin sections stained with saturated aqueous uranyl acetate followed by lead citrate were examined in a Siemens 1 electron microscope (Angus, Tzipori and Gray, 1982). Blocks for scanning electron microscopy were dehydrated in graded acetones, critical point dried and sputter coated in gold (Angus, Tzipori and Gray, 1982), for examination

in a scanning microscope (S180 Cambridge Instruments). Electron microscopy was carried out by Mr. E.W. Gray and Mr. J.D. Menzies, Moredun Research Institute.

(d) 3cm long segments from each site were removed aseptically for bacterial counts. The mucosal surface was scraped and 0.1g of scraping suspended and shaken in 9.9ml of PW. This was considered to constitute a 1/100 dilution.

(e) 3cm segments were taken from sites 2, 3 and 4 and stored at -20°C until assayed for lactase activity.

5. GENERAL METHODS

Giemsa staining for the detection of Cryptosporidium sp

Thin smears of faeces or faecal suspensions in PBS were made on glass microscope slides, air dried, fixed in methanol for 2 minutes then immersed in Giemsa stain (45ml distilled water, 2.5ml Giemsa stain (BDH), 2ml methanol and 0.3ml of 1.5% Na CO_3) for 1 hour. The smears were carefully rinsed in tap water, air dried and examined at X1,000 magnification by light microscopy.

Rotavirus ELISA

The method was that used by Fahey, Snodgrass, Campbell, Dawson and Burrells (1981). Analysis of faeces by ELISA was carried out by Miss I. Campbell, Moredun Research Institute.

Negative staining of faeces for electron microscopy

One drop of a 10% suspension of faeces in distilled water was applied to a formvar/carbon coated grid for 2 minutes, then the excess fluid was removed and the grid stained in 1% phospho-tungstic acid for 45 seconds. The grid was examined in an electron microscope after drying.

Single radial diffusion assay for bovine IgG, IgA and IgM (Mancini technique)

The technique of Fahey and McKelvey (1965), modified from Mancini, Carbonara and Heremans (1965), was used to estimate the immunoglobulin concentrations in bovine sera and fractionated whey samples.

Molten 2% Ionagar No. 2 in 1% NaN_3 was mixed with an equal volume of diluted pig anti-sheep IgG or rabbit anti-sheep IgA or IgM (kindly supplied respectively by Messrs. C. Gardiner and C. Burrells, Moredun Research Institute) in buffer (0.2M NaCl, 0.06M K_2HPO_4 pH 8.0) held at 60 °C. The optimal working concentrations of the anti-bovine immunoglobulins had been determined previously by Mr. C. Burrells. Twenty-five ml of agar was used for one 8cm x 12cm glass slide. Forty, 3mm diameter wells cut 1.2cm apart were filled (5 μ l/well) either with standard ovine IgG, IgA or IgM preparations (kindly supplied by Mr. C. Burrells) or test samples. Sera were diluted 1/20 in PBS and fractionated whey samples used undiluted. Gels were incubated for 24 hours for IgG reactions and 48 hours for IgA and IgM reactions in a humidified box at room temperature. The diameters of the precipitin rings which develop around the wells are proportional to the concentrations of specific antigen (immunoglobulin) contained by the samples. These concentrations were estimated from the calibration curve produced from standard immunoglobulin preparations.

Lactase assay

The method used was similar to that described by Dahlqvist (1964). 0.1g of mucosal scraping was homogenised at 4 °C in 10ml

of distilled water and the suspension clarified by centrifugation at 1500g for 10 minutes. Glucose produced by the enzyme lactase acting on the substrate lactose was assayed using a diagnostic kit (Sigma No. 510A Sigma Chemical Company, St. Louis, Missouri, USA).

(a) The enzyme assay

0.1ml of control or test sample was added to 0.1ml of lactose in 0.0625M malate buffer pH 6.0. The mixture was incubated at 37 °C for 60 minutes, then 0.8ml of distilled water was added and the suspension boiled for 10 minutes. The boiled mixture was then assayed for glucose. The positive control utilised 0.1ml lactase enzyme (Sigma, 88 units/ml); the negative control was the same, but the mixture was boiled as soon as the lactase was added to the lactose.

(b) Glucose assay

0.5ml of test sample was mixed with 5.0ml of freshly prepared enzyme colour reagent (100ml distilled water, 500IU of glucose oxidase (Aspergillus niger), 100 purpurgallin units of peroxidase (horseradish) and 50mg O-dianisidine dihydrochloride). All tubes were mixed thoroughly and the colour allowed to develop at 37 °C for 30 minutes. The colour intensity was read at 425nm within 30 minutes of the end of incubation. Glucose standards (6.25, 12.5, 25, 50, 100 and 1000mg/ml) were prepared and a negative control (0.5ml distilled water) were included in the test. The lactase activity was calculated thus;

$$\text{Lactase activity} = \mu\text{moles/min/g wet weight} = \frac{2 \times \mu\text{g glucose} \times 1000}{180 \times 60}$$

CHAPTER 3 SECTION A

THE PREVALENCE OF ENTEROTOXIGENIC E. COLI IN YOUNG CALVES IN SCOTLAND AND NORTHERN ENGLAND

INTRODUCTION

In 1893 Jensen reported mortality rates of 58 to 75% in calves from birth to 2 days of age. All deaths were from 'white scours' which were attributed to E. coli infections. Subsequent surveys of calf mortality in Britain have failed to identify the causes of neonatal diarrhoea. Jordan (1933) quoted an annual average mortality of 19.5% in calves, of which 40% was due to diarrhoea. Isolation of possible causative agents was not attempted, but it was suggested that E. coli played a major role. Lovell and Hughes (1935) examined 100 dead calves bacteriologically and concluded that 37 had died from colibacillosis. The E. coli strains isolated in this instance were not characterised in any way.

Lovell and Bradford-Hill (1940) concluded that the annual mortality of heifer calves was higher in Scotland (11.4%) than in England and Wales. Similarly Withers (1952) reported calf mortality to be 4.9 to 6.8% and 10.3 to 12.9% in England/Wales and Scotland respectively. Withers considered that colisepticaemia and 'white scours' accounted for 28% of the total number of deaths observed, and implicated E. coli as the commonest cause of death in the first 7 to 10 days of life.

In none of the works cited were the E. coli isolates tested for their ability to cause diarrhoea in calves, and it was not until studies by Smith and Halls (1967a) that some strains of E. coli

isolated from pigs and calves were shown to dilate ligated gut loops. This effect was shown to correlate with the ability of the strain to cause diarrhoea in vivo. These strains were said to produce enterotoxin and were therefore described as enterotoxigenic E. coli or ETEC.

The gut loop test revolutionised the search for ETEC, and surveys have been conducted over the last 15 years in the USA, Canada and Japan. (Acres, Laing, Saunders and Radostits, 1975; Myers, 1975; Myers and Guinee, 1976; Moon, Whipp and Skartvedt, 1976; Morin, Lariviere and Lallier, 1976; Sivaswamy and Gyles, 1976a; Isaacson, Moon and Schneider, 1978; Moon, McClurkin, Isaacson, Pohlenz, Skartvedt, Gillette and Baetz, 1978; Nakazawa, Nemoto, Ueda and Maruyama, 1981).

Calf ETEC not only produce an enterotoxin but also possess an adhesive antigen 'Kco' subsequently named K99 (Orskov, Orskov, Smith and Sojka, 1975). ETEC strains isolated from calves elaborate the heat stable enterotoxin active in infant mice (STa) but not the heat labile toxin (LT) active in the Y1 adrenal cell assay.

No surveys to date have established the prevalence of ETEC in calves in Britain. In this section of Chapter 3 data is presented on the prevalence of ETEC and other enteropathogens in the faeces of diarrhoeic and clinically normal calves from farms in Scotland and Northern England. Also presented are the results of a serological survey of K99 antibodies in the sera of cows and calves.

MATERIALS AND METHODS

Survey outline and samples taken

This survey was undertaken with the assistance of Veterinary

Investigation Centres (V.I.C.) located throughout Scotland and parts of Northern England. Veterinary surgeons encountering outbreaks of calf diarrhoea were requested to report these to the local V.I.C. who informed the Moredun Research Institute. Sample collections were made by V.I.C. staff or sometimes by Dr. D.R. Snodgrass.

Faecal samples were taken from calves that had recently become diarrhoeic and before treatment had been initiated. The survey was restricted to calves up to 21 days of age. On occasions on farm comparisons were made by sampling clinically normal calves. Faeces were collected and transported to the laboratory in sterile plastic or glass universal bottles. Five to 10ml of faeces were obtained which enabled several examinations to be performed on one sample. Samples were processed in the laboratory on the day of sampling, or, if sent by post up to 4 days after collection. The number of samples taken from each farm ranged from 2 to 22.

Isolation of *E. coli* and examination for K99, STa, LT and VT

The methods used were those described in Chapter 2. In general 5 *E. coli* colonies grown from each calf faeces sample were tested for K99 antigen and 3 of these 5 isolates for STa and LT. The VT test was only carried out on K99⁺ isolates.

Positive findings in the LT test were confirmed by a neutralisation assay in which 100μl of culture supernate was incubated at 37 °C with 100μl of diluted rabbit anti-cholera toxin (donated by Dr. S. Van Heynigen, University of Edinburgh) for 1 hour before use in the normal test.

Calf ligated loop test

The method was adapted from that described by Myers, Newman,

Warren, Catlin and Anderson (1975).

Strains of E. coli were grown overnight in 10ml TSB at 37 °C on a rotary shaker. The bacterial growth was centrifuged at 1600g for 20 minutes at 4 °C and the pellet resuspended in 2ml of fresh TSB. The suspensions contained approximately 10^{10} cfu/ml.

Jersey or Ayrshire colostrum fed calves 5 to 10-days old were used. They were deprived of feed and water 24 to 36 hours before start of the experiment.

All surgical procedures were performed under halothane-nitrous oxide anaesthesia. A 15cm ventro-dorsal incision was made on the right flank mid-way between the final rib and the ischial protuberance; the ileo-caecal junction was then located.

The first ligature was placed 2 to 3 metres proximal to the ileo-caecal junction. Forty to 60 isolated loops 7 to 10cm long were prepared using braided nylon suture (3 metric) ligatures at each end. These loops were proximal to the first ligature and separated from each other by 3 to 5cm lengths of intestine. Loops were inoculated with 2ml of an appropriate bacterial suspension through a 26 gauge needle. Two of each set of 10 loops were used as controls, one of which was inoculated with the K12 (enterotoxin negative) E. coli strain and one with strain B44 (enterotoxin positive).

Calves were injected intravenously (I.V.) with 1500ml of sterile Ringers solution during surgery which lasted approximately 2 hours.

In early assays anaesthesia was maintained for 8 hours after termination of surgery, then the calves were killed by I.V. injection of sodium pentobarbitone (200mg/ml, May and Barker Ltd, Dagenham, England) and the test section of small intestine removed intact.

Under these conditions, using 3 calves, variable results were obtained with the control enterotoxin positive loop dilating strains. In later assays, therefore, calves were allowed to recover from anaesthesia and given 0.1ml xylazine (Rompon, 2% solution Bayer UK, Bury St Edmonds, Suffolk, England) intramuscularly (I.M.). Analgesia was maintained by I.M. injection of pethidine (50mg/ml, Evans Medical, Speke, Liverpool, England) every 4 to 6 hours. Twenty to 24 hours post surgery calves were killed with barbiturate, the small intestine removed and the mesentery partially dissected away. The length and volume of fluid contained in each loop was measured and the volume/length ratio calculated (ml/cm). A value >1ml/cm was considered a positive reaction provided that the relevant control loops had responded correctly. Each isolate was tested in at least 2 loops, and where possible, in different calves.

Biochemical tests on isolates

Two hundred and eighty nine isolates were tested either by the Sensititre test (Seward Laboratories, 149 isolates) or Micro-D (General Diagnostics, Morris Plains, New Jersey, 140 isolates) to confirm that lactose fermenting colonies isolated were E. coli.

Serotyping of isolates

Representative K99⁺ E. coli isolates from each outbreak were sent to Drs I and F Orskov (Statens Serum Institut, Copenhagen, Denmark) who kindly established the respective O, K and H antigens.

Antibiotic sensitivity of isolates

A random sample of 33 K99⁻ and 27 K99⁺ E. coli isolates were tested for their in vitro sensitivities to 7 antimicrobial agents using Mastring-S discs (Mast Laboratories, Liverpool, England):

streptomycin (10µg), gentamicin (10µg), ampicillin (10µg), polymyxin B (100 units), chlorotetracycline (25µg), chloramphenicol (25µg) and neomycin (30µg).

Isolates were grown for 3 hours at 37 °C in TSB, and then inoculated to provide confluent growth on to Dextrose Sensitivity Test agar (Oxoid, Cm 261) using a swab. The Mastring-S discs were applied and the plates incubated at 37 °C for 18 hours.

Antibiotic sensitivities were assessed from the diameter of the zone of inhibition of growth around the discs with reference to data presented in the Diamed Diagnostics Ltd Laboratory Manual; 'Susceptibility Testing Products' (1981). Isolates with reduced or no zone of inhibition were considered to be resistant in the test, although it should be recognised that reduced zones of inhibition may be an influence of inoculum weight and the antibiotic may still be active at therapeutic levels.

Examination of moribund calves

Moribund calves bought from 2 affected herds were necropsied as described in Chapter 2.

Source of sera and method of examination for K99 antibodies

Sera from 304 calves, 1 to 6 months of age, were tested. The sera had been submitted to the Virology Section of Moredun Research Institute during 1981 for diagnostic purposes unconnected with this investigation.

Sera were taken from 179 cows from 13 Scottish herds in which calf diarrhoea had been considered a problem. Eleven to 30 samples were taken from each herd.

All sera were tested by sandwich ELISA as described in Chapter

4, Section A.

Examination for other enteropathogens

Faeces were examined for rotavirus by ELISA, for coronavirus by haemadsorption-elution-haemagglutination assay (HEHA) (van Balken, de Leeuw, Ellens and Straver, 1979) and for Cryptosporidium sp by the Giemsa staining method (Chapter 2). Faeces were also examined by electron microscopy for enteric viruses using negative staining (Chapter 2). These procedures were carried out by Dr. Snodgrass, I. Campbell, P. Richardson, E.W. Gray and J. Menzies.

RESULTS

K99 antigen testing

Of the 1529 E. coli isolates tested for K99 antigen, 88 (5.75%) were positive. The K99⁺ isolates were obtained from 8 of the 70 (11.43%) herds sampled and this constituted isolation of K99⁺ E. coli from 23 of 362 calves (6.35%). However, 56 of the 362 calves were clinically normal at the time of sampling, all 256 isolates from these clinically normal calves were K99⁻. Thus, K99⁺ E. coli were isolated from 23 of 306 diarrhoeic calves (7.52%, $P = 0.07$) which gives an isolation rate of 88 of 1273 isolates (6.91%).

All 88 isolates were shown to be K99⁺ after culture on minca-Isovitalex agar at 37 °C either by using K99 ELISA (Chapter 4, Section A) or by using the slide agglutination test employing the 5 antisera described in Chapter 2. All K99⁺ isolates (Table 3.1) failed to produce K99 antigen when grown on minca-Isovitalex agar at 15 to 20 °C.

STa testing

The 685 E. coli isolates tested in the infant mouse assay for

Table 3.1 Characteristics of K99⁺ E. coli isolated from diarrhoeic calf faeces

| Farm | Calf No | Number positive/ Number tested | | | | | Serotype | Antibiotics to which strains were resistant ^a |
|------|---------|-----------------------------------|-----|-----|-----|-----|-------------------------|--|
| | | K99 | STa | LT | VT | Hly | | |
| 1 | E346 | 5/5 | 3/3 | 0/3 | NT | + | 08:K85:H27 | S |
| | E349 | 5/5 | 3/3 | NT | NT | + | | NT |
| | E350 | 5/5 | 3/3 | NT | NT | + | | NT |
| | E351 | 5/5 | 3/3 | 0/3 | NT | + | 08:K85:H27 | NT |
| | E352 | 3/5 | 1/1 | NT | NT | + | | NT |
| | E354 | 2/5 | 1/1 | NT | NT | + | 08:K85:H27 | NT |
| | E355 | 5/5 | 3/3 | 0/3 | NT | + | | P, S |
| 2 | 9051/1 | 3/3 | 3/3 | 0/3 | NT | + | | S, A, Ct |
| | 9051/2 | 3/3 | 3/3 | 0/3 | 0/1 | + | | S, A, Ct |
| | 9051/3 | 3/3 | 3/3 | 0/3 | NT | + | | NT |
| | 9076/1 | 3/3 | 3/3 | 0/3 | NT | + | 08:K85:H27 | S, Ct |
| | 9076/2 | 3/3 | 3/3 | 0/3 | NT | + | | NT |
| 3 | B/H | 5/5 | 3/3 | 0/3 | 0/1 | + | 08:K85:H27 | S, A, Ct, C |
| 4 | B2625/3 | 5/5 | 3/3 | 0/3 | 0/3 | + | 08:K ⁺ :H? | S, A |
| | B2625/4 | 5/5 | 3/3 | 0/3 | 0/3 | + | | S, N |
| | B2625/5 | 5/5 | 3/3 | 0/3 | 1/3 | + | | S, N |
| | B2625/6 | 5/5 | 3/3 | 0/3 | 0/3 | + | | S, N |
| 5 | B2649/1 | 3/3 | 3/3 | 0/3 | 0/3 | + | | S |
| | B2649/3 | 3/3 | 3/3 | 0/3 | 0/3 | + | 08:K85:H ⁻ | S |
| 6 | D650/1 | 5/5 | 3/3 | 0/3 | 0/3 | + | 08:K85:H? | S |
| 7 | D679/1 | 5/5 | 3/3 | 0/3 | 0/3 | - | 0101:K28:H ⁻ | S, A, Ct |
| 8 | C56/1 | 1/1 | 1/1 | 0/1 | 0/1 | - | 0141:K85ab:H? | S, A |
| | C56/2 | 1/1 | 1/1 | 0/1 | 0/1 | - | 0141:K85ab:H? | S, A |

STa; stable toxin active in infant mice.

LT; labile toxin.

VT; vero cytotoxin.

Hly; haemolysis produced on sheep blood agar.

NT; not tested.

H?; only weakly motile.

S = Streptomycin G = Gentamicin A = Ampicillin P = Polymyxin B
C = Chloramphenicol Ct = Chlorotetracycline N = Neomycin

STa activity came from 311 calves, of which 279 (605 isolates) were diarrhoeic and 32 (80 isolates) were clinically normal. The distribution of gut/body weight ratios for all isolates tested are shown in Figure 3.1 and control test mice are shown in Figure 3.2.

None of the isolates from clinically normal calves were STa⁺, whereas 61 (10.1%) of those from diarrhoeic calves were STa⁺. The mean gut/body weight ratio produced by all STa⁺ isolates was 0.1007 ± 0.003 compared with a mean ratio of 0.0574 ± 0.001 for 61 randomly selected negative isolates. The STa⁺ isolates were K99⁺ and came from 23 diarrhoeic calves. Thus there was complete correlation between possession of K99 antigen by an isolate and its ability to produce STa.

LT testing

Of 743 isolates tested in the Y1 mouse adrenal tumour cell assay, 8 isolates, which originated from 5 farms, caused cell rounding (Figure 3.3). Six of these were derived from the 110 isolates tested from clinically normal calves and 2 from the 633 isolates tested from diarrhoeic calves. The active factor was heat labile (100 °C for 10 minutes) but cell rounding was not neutralised rabbit anti-cholera toxin serum. Immunologically therefore, the effect induced by these isolates was not due to classical LT. The nature of the factor that caused rounding of Y1 adrenal cells was not identified.

Five representative isolates which showed this Y1 cell-effect (one isolate from each farm) were serotyped. Two were found to be mixed cultures (036:K⁻:H? spontaneous agglutination and 02:K⁻:H8; and 015:K⁻:H? spontaneous agglutination and 0116:K⁻:H⁻) and 3

Figure 3.1 Infant mouse assay for STa; distribution of the gut/
body weight ratios of mice inoculated with bacteria
free culture supernates from the growth of 685 calf
E. coli isolates.

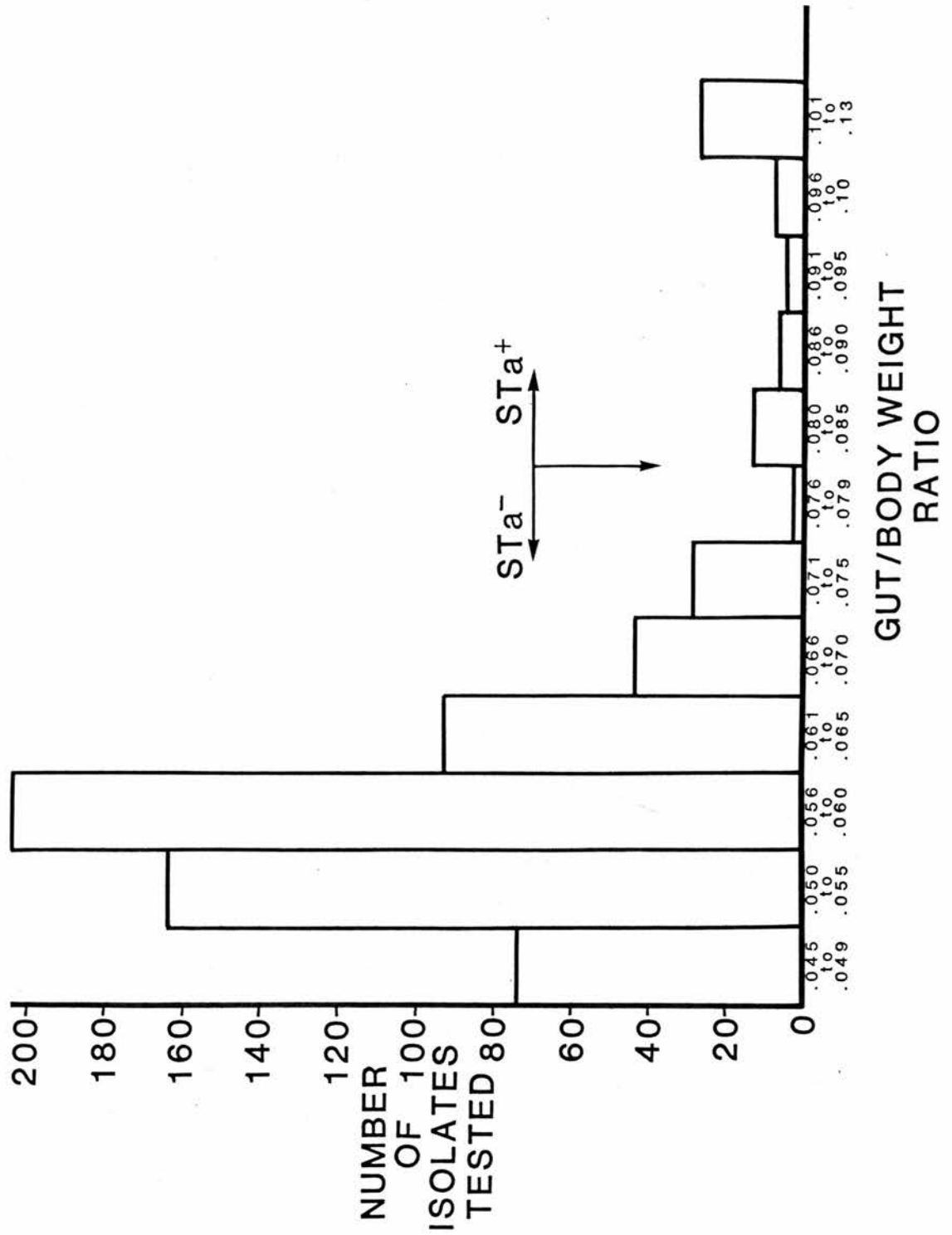


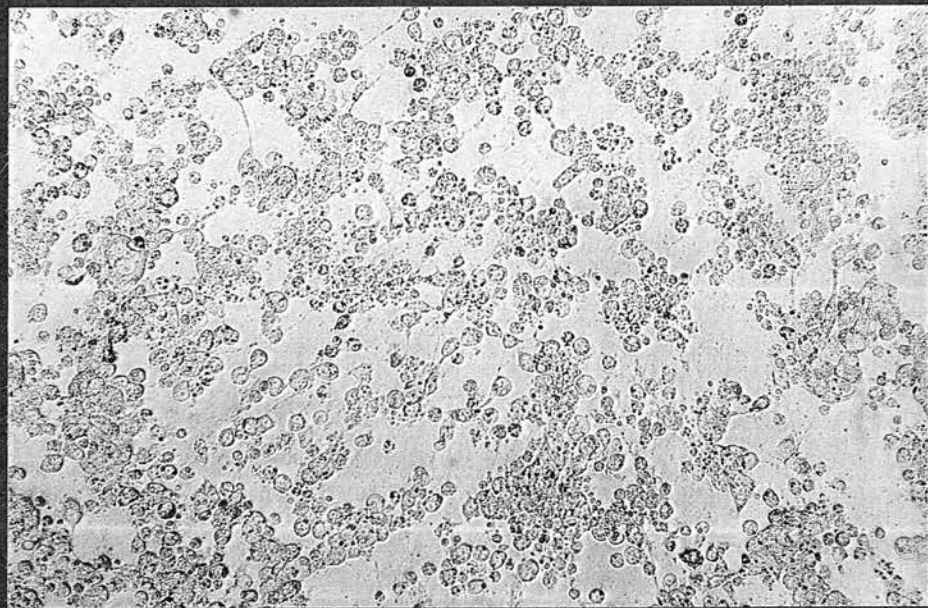
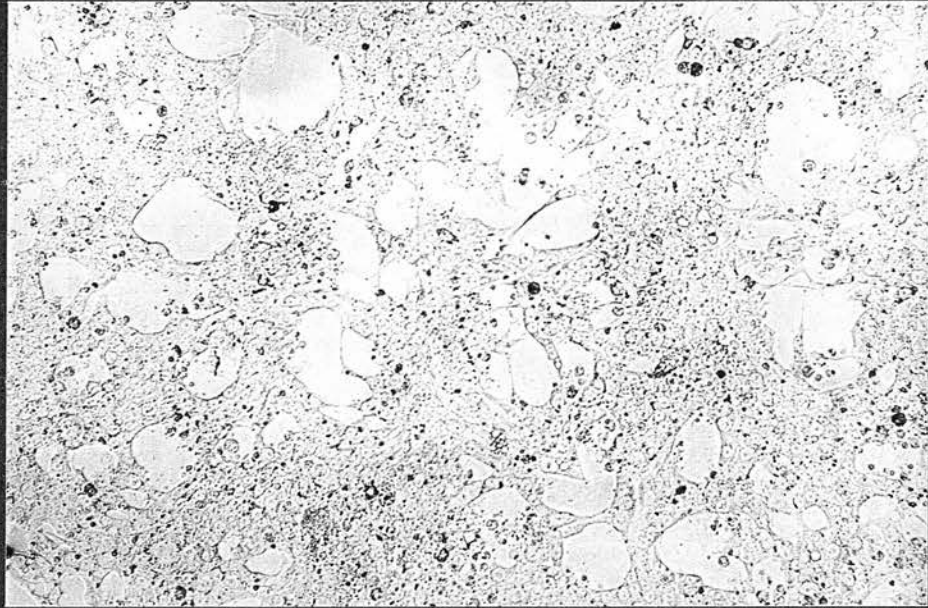
Figure 3.2 Infant mouse assay for heat stable toxin, STa.

- (a) Mouse 4 hours post inoculation with a bacteria free culture supernate containing STa (Strain EC6).
Note the dilated intestine.**
- (b) Mouse 4 hours post inoculation with a bacteria free culture supernate not containing STa.**



Figure 3.3 Y1 mouse adrenal tumour cell assay for heat labile toxin, LT.

- (a) Cell monolayer 20 hours post inoculation with a bacteria free culture supernate not containing LT.
(x150, unstained)
- (b) Cell monolayer 20 hours post inoculation with a bacteria free culture supernate containing LT (Strain EC1). Note cell rounding. (x150, unstained)



monocultures (015:K⁻:H21; 015:K⁻:H21 and 07:K⁻:H31).

None of the K99⁺ isolates shown in Table 3.1 produced labile toxin.

Vero cytotoxic factor testing

Of 28 K99⁺ isolates tested only one was positive in this test (Figure 3.4). This isolate was from farm 4 (Table 3.1) and was shown to produce K99 antigen and STa.

Calf ligated loop testing

All 16 K99⁺ STa⁺ LT⁻ isolates tested in calf gut loops evoked positive reactions in one or two loops (1 to 8ml/cm, Table 3.2). All 19 randomly selected K99⁻ STa⁻ LT⁻ isolates were consistently negative (<1ml/cm) in the test. Positive and negative gut loop reactions are shown in Figure 3.5.

Six of the 8 isolates that caused rounding of Y1 adrenal cells were examined in the calf ligated loop test and found to be negative.

Serotyping analysis and haemolysin production of isolates

Eleven representative K99⁺ isolates from 8 farms (Table 3.1) were serotyped. Five different serotypes were found, 08:K85:H27 (5 isolates from farms 1 to 3); 08:K⁺:H? (1 isolate from farm 4); 08:K85:H⁻ (2 isolates from farms 5 and 6); 0101:K28:H⁻ (1 isolate from farm 7) and 0141:K85ab:H? (2 isolates from farm 8).

Eight of the 11 serotyped isolates were haemolytic (Hly⁺) on sheep blood agar, the haemolytic activity being associated with the 08 serogroup isolates. The 0101 and 0141 serogroup isolates were non-haemolytic (Hly⁻).

Table 3.2 Calf ligated gut loop testing of K99⁺ E. coli

| Isolate | Ratio of fluid in loop to length of loop (ml/cm) | | | |
|---------|---|--------|------------|------------|
| | Calf 1 | Calf 2 | Calf 3 | Calf 4 |
| B44 | 1.43 ^a | 1.04 | 3.68 | 2.39 |
| K12 | 0 | 0 | 0 | 0 |
| E346 | 1.44 | NT | NT | NT |
| E351 | 3.10 | NT | 5.0 | NT |
| E355 | 1.55 | NT | 2.86 | NT |
| 9051/1 | 1.05 | NT | 1.67 | NT |
| 9051/2 | NT | NT | NT | 3.78, 3.75 |
| 9051/3 | NT | 0.79 | 1.05 | NT |
| 9076/1 | 0.96 | 2.3 | NT | NT |
| B/H | 0.84 | 1.22 | NT | NT |
| B2625/3 | NT | NT | 1.67, 1.88 | NT |
| B2625/4 | NT | NT | NT | 1.63 |
| B2625/5 | NT | NT | NT | 3.56, 3.36 |
| B2649/1 | NT | 2.20 | 5.00 | NT |
| B2649/3 | NT | 1.30 | 8.30 | NT |
| D650/1 | NT | NT | NT | 3.13 |
| D679/1 | NT | NT | NT | 3.50, 1.25 |
| C56/2 | NT | NT | NT | 3.20 |

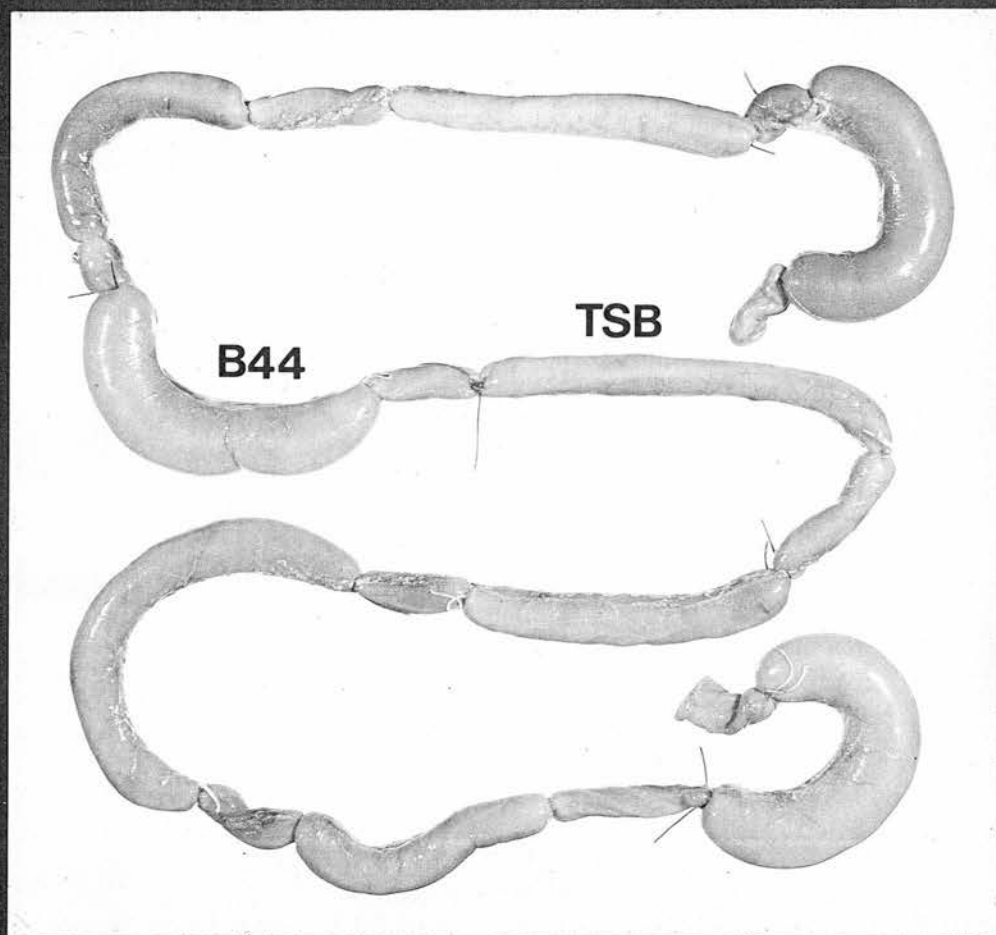
NT; not tested.

a; values given are an average of at least 2 loops.

Figure 3.4 Vero cell assay for Vero cytotoxic factor, VT. Cell monolayer 48 hours post inoculation with a bacteria free culture supernate containing VT (Strain H19). Note dead cells on the surface of the monolayer. (x150, unstained)



Figure 3.5 Calf ligated loop test. Gut loops 20 to 24 hours post inoculation with test isolates. Note dilated and normal loops with positive (B44) and negative control (TSB) loops.



In total 86 of 1529 isolates were Hly⁺ (5.6%) and 1443 were Hly⁻ (94.4%). Eighty-one of the Hly⁺ and 7 of the Hly⁻ isolates were K99⁺. On a farm basis Hly⁺ K99⁺ E. coli were isolated from 6 of the 8 farms from which K99⁺ E. coli were obtained. Only one calf excreted Hly⁺ K99⁻ E. coli.

Biochemical testing of isolates

Of the 289 isolates tested 286 were confirmed as E. coli, 2 were found to be Serratia spp and 1 Citrobacter spp. Salmonella typhumurium was isolated from the faeces of one calf.

Antibiotic resistance of K99⁺ and K99⁻ E. coli strains

The results are summarised in Table 3.3. The K99⁻ E. coli isolates tended to be more resistant than the K99⁺ isolates for most antibiotics tested. Significant differences to certain antibiotics was seen for chloramphenicol (P = 0.07) and chlorotetracycline (P = 0.001) in which K99⁻ isolates were more resistant than K99⁺ isolates. The K99⁺ isolates were significantly more resistant to streptomycin (P = 0.005) than the K99⁻ isolates. Gentamicin and polymyxin B were active against all K99⁺ isolates and most K99⁻ isolates.

Serological survey for K99 antibodies by ELISA

Seven of 179 cow sera tested (3.9%) were positive for K99 antibodies. Four of these cows came from one farm and the remainder from 3 separate farms.

Nine of the 304 calf sera tested (2.96%) were positive for K99 antibodies. Four of these came from one farm and the remainder from 5 separate farms.

Table 3.3 Antibiotic resistance of K99⁺ and K99⁻ E. coli
isolated from the faeces of calves

| Antibiotic | Percentage of strains demonstrating resistance | |
|--------------------|--|--|
| | K99 ⁻ <u>E. coli</u> (33 isolates) | K99 ⁺ <u>E. coli</u> (27 isolates) |
| Streptomycin | 69.7 | 100 ^b |
| Gentamicin | 0 | 0 |
| Ampicillin | 42.4 | 30 |
| Polymyxin B | 3.0 | 0 |
| Chlorotetracycline | 75.5 | 22.2 ^c |
| Chloramphenicol | 24.2 | 3.7 ^a |
| Neomycin | 26.7 | 37.5 |

a; P = 0.07 by Chi-square test.

b; P = 0.005 by Chi-square test.

c; P = 0.001 by Chi-square test.

Other enteropathogens detected

The ELISA and electron microscopic examination detected rotavirus in 90 diarrhoeic calves (29.4%) and 6 clinically normal calves (10.7%; $P < 0.01$). Seventeen diarrhoeic calves (5.6%) and 4 clinically normal calves (1.3%) were shown to be excreting coronavirus (P not significant) by the HEHA technique. Cryptosporidium sp was present in the faeces of 43 diarrhoeic calves (14.1%) and 4 clinically normal calves (7.1%; P not significant).

Including the ETEC isolations at least one enteropathogen (rotavirus, coronavirus or Cryptosporidium sp) was detected in 148 of 306 diarrhoeic calves (48.3%) and 14 of 56 clinically normal calves (25.0%).

Mixed infections

Twenty-one diarrhoeic calves (6.9%) had mixed infections, comprising ETEC and rotavirus (3 calves), coronavirus and rotavirus (5 calves), Cryptosporidium sp and rotavirus (12 calves) and Cryptosporidium sp, coronavirus and rotavirus (1 calf).

Age distribution of ETEC infections

All ETEC infections occurred in calves under 3 days of age, diarrhoea commonly starting within 24 hours of birth. On 6 farms (farms 3 to 8, Table 3.1) ETEC were the only enteropathogens isolated. Two farms (farms 1 and 2, Table 3.1) had concurrent ETEC and rotavirus infections in some of the calves examined. The dual infected calves became diarrhoeic between 24 and 72 hours of age.

Examination of 2 moribund calves

Calf (a)

E. coli isolates from this 5-day-old calf were K99⁻ ST⁻ LT⁻.

One isolate was serotyped as O153:K⁻:H4 and was negative in the ligated calf gut loop test. Bacterial counts taken from 3 sites in the small intestine (duodenum, jejunum and ileum) gave viable counts (cfu per g wet weight) of 10^5 , 6.87×10^8 and 4.6×10^8 respectively. Histological examination showed mild stunting and fusion of villi in the jejunum and mid gut but neither coronavirus nor rotavirus were detected in the faeces or caecal contents. On the basis of these findings no diagnosis could be made.

Calf (b)

This calf, from farm 3, was 48 hours old when killed. A haemolytic K99⁺ E. coli had been isolated from the faeces (isolate B/H, Table 3.1). Mucosal scrapings from the duodenum, jejunum and ileum gave viable counts (cfu/g wet weight) of 10^4 , 6×10^7 and 10^9 respectively. Indirect immunofluorescent staining of cryostat sections from the above sites using adsorbed K12:K99 antiserum revealed extensive bacterial adherence to epithelial cells in the posterior small intestine. Histological examination confirmed the bacterial adherence but morphological alterations of the gut were not apparent. Neither coronavirus nor rotavirus were detected in the faeces.

DISCUSSION

K99⁺ ETEC were isolated from the faeces of 7.52% of diarrhoeic calves on 11.43% of the farms studied in this survey. There was complete correlation between K99 production, elaboration of STa and dilation of calf ligated loops by ETEC.

Examination of 1529 E. coli isolates from diarrhoeic and normal calves for K99 antigen detected 88 K99⁺ isolates, all from

diarrhoeic calves. This isolation rate differed significantly ($P = 0.07$) from that of normal calves. Although ETEC were not found in clinically normal calves in this study, Isaacson, Schneider and Moon (1978) reported that 17% of E. coli isolated from normal calves were ETEC compared with 47% from diarrhoeic animals, and Nakazawa, Nemoto, Ueda and Maruyama (1981) reported isolation results of 1.2% and 18.0% respectively.

Previous studies have not shown complete correlation between the presence of K99 antigen and the production of STa, as some STa producing isolates were shown to be K99⁻ eg only 95.6%, 76.0% and 86.4% ETEC were found to be K99⁺ by Guinee, Jansen and Agterberg (1976), Moon, Whipp and Skartvedt (1976) and Isaacson, Schneider and Moon (1978) respectively. The discrepancy could be due to the effectiveness of the test procedures for K99 antigen. In this present study K99 tests were made on isolates after overnight growth on minca-Isovitalex agar, a technique which is reported to increase the number of K99⁺ isolations made compared with minca agar (Guinee, Veldkamp and Jansen, 1977). There is however evidence to suggest that serial passage in TSB before culturing on minca-Isovitalex agar increases the detection of K99⁺ E. coli (Isaacson, Schneider and Moon, 1978). The serial passage method is time consuming and was not used both for this reason and because it was considered that K99⁺ ETEC isolates missed by this technique would be detected by the infant mouse assay. Because of the complete correlation between K99 and STa there is no evidence from this work that the test system does not detect the majority of K99⁺ E. coli or that atypical ETEC commonly occur in Scotland and Northern England.

Adhesive antigens on E. coli could additionally have been detected using isolated brush borders and villi; Girardeau (1980) demonstrated that 36 of 84 E. coli isolates from diarrhoeic calf faeces attached to isolated calf villi in vitro, and that this attachment was inhibited by K99 antiserum. An additional 33 isolates attached to villi in vitro, but this attachment was not inhibited by K99 antiserum. The villus adhesion test can therefore be used to screen adhesins other than K99 antigen.

K99⁺ isolates gave high gut/body weight ratios in the infant mouse assay. The assay is widely used by many workers for STa detection, although one has to be aware that STb (Gyles, 1979), produced by pig ETEC may also be produced by calf strains and thus such strains will not be detected in this assay. Nakazawa, Nemoto, Ueda and Maruyama (1981) reported that some K99⁺ E. coli did not react in the infant mouse assay, hence it is possible that either STb may have been produced, the isolates had not produced enough toxin to give a positive reaction or they were non-toxin producers. The infant mouse assay needs to be replaced as a screening method for STa as it is time consuming and costly in animals. Two radio-immuno-assay procedures have been recently developed to detect STa (Gianella, Drake and Luttrell, 1980; Frantz and Robertson, 1981). These tests apart from being highly sensitive, reduce the need for excessive use of experimental animals.

All isolates tested for LT in the Y1 adrenal cell assay were found to be negative, although 8 isolates were shown to cause cell rounding which on further investigation, was not inhibited by anti-cholera toxin. Isolates such as these have been found in the USA

(Dr. H.W. Moon, personal communication). These strains were not considered to be LT producers as LT can be neutralised by anti-cholera toxin (Clements and Finklestein, 1978) and they did not dilate calf ligated gut loops. The absence of any LT⁺ isolates in this survey is in general agreement with other surveys (Moon, Whipp and Skartvedt, 1976; Isaacson, Schneider and Moon, 1978; Nakazawa, Nemoto, Ueda and Maruyama, 1981), although one LT⁺ isolate was found by Isaacson, Schneider and Moon, (1978) and 6 LT⁺ isolates by Moon, Whipp and Skartvedt, (1976). However in contrast to these microbiological findings serum antibodies to LT were found in 87% of calves (Dobrescu, 1979) and 38% of cows (Whipp and Donta, 1976). It is possible that calves and cows may be exposed to organisms producing a substance which is serologically similar to LT, or present in vitro tests may be unable to detect LT⁺ isolates. Bacteria other than E. coli such as Citrobacter spp (Acres, Laing, Saunders and Radostits, 1975) and Salmonella spp (Jiwa, Krovacek and Wadstrom, 1981) have been reported to produce STa or LT and these organisms could account for the presence of LT antibodies in bovine sera.

Twenty-eight K99⁺, STa⁺, LT⁻ E. coli isolates were examined for the Vero cytotoxic factor. Only one isolate was positive, which is a similar isolation frequency to that reported by Kashiwazaki, Ogawa, Isayama, Akaike, Tamura and Sakasaki (1980) and Dr. H.W. Smith (personal communication). The role of the Vero cytotoxic factor in the pathogenesis of calf diarrhoea is not known, but its low association with ETEC suggests that its presence may not be significant.

The most commonly used test for confirming the enterotoxigenicity of an E. coli isolate is probably the calf ligated loop test. This test was used in most of the early surveys on calf diarrhoea (cited in the introduction to this Chapter). In this present study there was complete correlation between K99 antigen and STa production by an isolate and its ability to dilate ligated loops. This may be the most accurate in vivo test for testing the enteropathogenicity of an E. coli isolate other than the experimental challenge of calves under 24 hours of age.

Limited serotyping revealed that K99⁺ ETEC were associated with the 08 serogroup and all 08 isolates were haemolytic (Hly⁺). Haemolysin production by E. coli has not been correlated with calf ETEC, nor has it been shown to be a virulence character. Only 1 of 10 and 4 of 56 ETEC examined were found to be Hly⁺ by Smith and Halls (1967a) and Sivaswamy and Gyles (1976b) respectively.

The comparison of K99⁺ and K99⁻ E. coli isolates for their resistance to 7 antibiotics (Table 3.3) showed that, with one exception, K99⁻ isolates were the more resistant. ETEC isolates from the same farm generally showed similar resistance patterns, but between farms resistance patterns were different. Little information on the antibiotic resistance of ETEC is available, although a previous survey (Sivaswamy and Gyles, 1976b) showed no difference between ETEC and non-ETEC. Multiple antibiotic resistance should not therefore be used as a marker of ETEC in veterinary diagnostic laboratories until further information is available.

The survey of cow and calf sera confirms the low prevalence of K99⁺ E. coli. The K99 antibodies detected in 2.96% of calf sera could represent either actively or passively acquired antibody

since calves of 1 to 6 months of age were sampled (see Chapter 4, Section B). Serology could clearly be used to detect herds in which K99⁺ ETEC were present and potentially pathogenic. Previous studies in Holland (Ellens, de Leeuw and Rozemond, 1978) and Israel (Kornitzer and Tamarin, 1979) showed very few cows and calves to have antibodies to K99.

On 8 farms, calves under 24 hours of age were found to be infected with ETEC, and on 2 farms rotavirus was also detected in some of the ETEC infected calves. The ages of calves infected by ETEC are consistent with previous reports (Smith and Halls, 1967a; Acres, Laing, Saunders and Radostits, 1975; Morin, Lariviere and Lallier, 1976; Moon, McClurkin, Isaacson, Pohlenz, Skartvedt, Gillette and Baetz, 1978; Lariviere, Lallier and Morin, 1979). One calf (calf (b)) was necropsied at 2 days of age and bacterial counts in the posterior small intestine together with immunofluorescence studies confirmed that the calf had enterotoxigenic colibacillosis. This procedure has the merit that it associates the isolated enteropathogen with the disease, but a disadvantage is that findings at necropsy may not reflect the disease at the initiation of infection. A diagnosis was not achieved for a further calf (calf (a)), possibly because the enteropathogen was undetected by our methods or because the infection had been eliminated by the time necropsy was performed.

Rotavirus, coronavirus and Cryptosporidium sp were isolated more frequently from diarrhoeic than clinically normal calves, although these differences were only significant for rotavirus ($P = 0.01$). The isolation of ETEC from diarrhoeic calves was significant at $P = 0.07$. Mixed infections were found in 6.9% of

calves studied, the majority of them involving rotavirus and Cryptosporidium sp.

Recent experimental work has associated Campylobacter spp with enteritis in calves (Al-Mashat and Taylor, 1980a; 1980b; 1981; Firehammer and Myers, 1981). Preliminary results for the isolation of Campylobacter spp from calf faeces in this and an extended survey has shown that these organisms have been isolated with equal frequency from diarrhoeic and normal calves (Terzolo and Lawson, unpublished results). Further work is clearly required to indicate the part that these organisms play in naturally occurring enteric diseases of calves.

In conclusion, approximately 50% of calf diarrhoea cases had a possible infectious cause and 7.5% of diarrhoeic calves were infected with ETEC. These findings are lower than those reported from Canada and USA in which 20 to 40% of diarrhoeic calves studied were infected with ETEC.

CHAPTER 3 SECTION B

NEONATAL CALF DIARRHOEA SURVEY IN LIBYA

INTRODUCTION

Extremely high calf mortality was experienced on several farms in the Tripoli Dairy Cattle Project, Libya, mainly due to diarrhoea. The problem was briefly surveyed by Higgins (1981), who confirmed the initial clinical assessment and suggested that an investigation be initiated to identify the specific aetiological agents involved. This project was undertaken by Dr. D.R. Snodgrass and the author. Our investigations extended from 17th October, 1981 to 7th November, 1981.

Most of the laboratory work was performed by the author using the primitive laboratory facilities and equipment provided. Field visits initially made by Dr. Snodgrass were repeated by the author to collect further samples.

The investigation provided an opportunity to assess diagnostic techniques, used at the Moredun Research Institute, under difficult field conditions and to compare calf diarrhoea in Libya with that in Scotland and Northern England.

MATERIALS AND METHODS

Sampling

Fourteen farms were involved in the survey. Unfortunately, most of the farms were at the end of their calving period by October and only 53 diarrhoeic and 22 clinically normal calves were sampled. Calves were generally sampled on the first observed day of diarrhoea, before treatment was administered. Faeces collected were examined

within 48 hours of collection for K99⁺ E. coli, rotavirus, coronavirus, Cryptosporidium sp and Salmonella spp. In general 4 or more calves were sampled on each farm.

Isolation of E. coli and K99 antigen testing

The methods used are described in Chapter 2. Isolates typed as K99⁺ were stored on Dorset egg slopes and transported to the Moredun Research Institute under import licence. These isolates were examined again for K99 antigen using the antisera described in Chapter 2, and were confirmed as E. coli using the MICRO-ID test. Antibiotic sensitivity testing was carried out on K99⁺ isolates using the Mastring-S system described in Chapter 3, Section A, and serotyping of selected isolates was carried out by Drs F. and I. Orskov (Statens Seruminstitut, Copenhagen, Denmark).

STa, LT, VT and ligated gut loop tests on E. coli isolates

These tests were carried out on a limited number of K99⁺ isolates by the methods described in Chapter 2 and Chapter 3, Section A.

Rotavirus ELISA

The method used was similar to that described by Fahey, Snodgrass, Campbell, Dawson and Burrells (1981), except that blocking sera were incorporated to check the specificity of the test. The hyperimmune sera used for blocking purposes were raised against ovine rotavirus (positive blocking serum) and bovine coronavirus (negative blocking serum) and used at a dilution of 1/20 in PBS.

Coronavirus ELISA

The method had been developed by D. Reynolds (Institute

for Research on Animal Diseases, Compton, England) specifically for bovine coronavirus.

Microtitre plates (Linbro, E.I.A. microtitration plates, Flow Laboratories) were coated overnight at 37 °C (100µl/well) with the globulin fraction of an anti-bovine coronavirus serum diluted 1/250 in 0.05M sodium carbonate buffer pH 9.6. The plates were then washed 3 times in 0.05% Tween 20 in PBS.

Ten per cent faecal suspensions in PBS were added in 100µl volumes to duplicate wells and incubated at 37 °C for 3 hours. A known positive faeces was included in the test as a control. Plates were washed as before, then 100µl of PBS was added to one of the duplicate wells and 100µl of a 1/40 dilution of an anti-bovine coronavirus (pig) serum to the other.

Anti-bovine coronavirus serum labelled with horseradish peroxidase diluted 1/500 in PBS containing 0.05% Tween 20 and 5% foetal calf serum was added in 100µl volumes to each well and incubated at 37 °C for 1 hour.

Plates were washed as before and 100µl of substrate was added to all wells. The substrate used was 10mg of recrystallised 5-amino-salicylic acid in 10ml of stock buffer (diluted 1/10) to which hydrogen peroxide was added to a concentration of 0.005% immediately before use. Stock buffer comprised 0.1M NaH_2PO_4 ; 0.1M Na_2HPO_4 and 0.1M EDTA added in a ratio of 50:55:1 respectively and adjusted to pH 6.8. Plates were incubated at 37 °C for at least 1 hour, then read by eye. If reduced colour was observed in the blocked compared with the unblocked well, the sample was scored positive.

Cryptosporidium sp

The method used for staining faecal smears by Giemsa's method is described in Chapter 2.

Zinc sulphate turbidity test (ZST)

The method was adapted from that used by McEwan, Fisher, Selman and Penhale, (1970). Serum was added in 100 μ l volumes to 0.9ml distilled water and 5.0ml of 0.001M zinc sulphate solution. The precipitate formed was compared with a standard positive and negative serum treated in the same way. Since a spectrophotometer was not available no attempt was made at quantification, samples being scored adequate (obvious precipitation) or inadequate (slight or no precipitation) for immunoglobulin content.

RESULTS

Calf mortality and enteropathogens detected

Calf mortality on the 14 farms studied is summarised in Table 3.4. The enteropathogens demonstrated in the faeces of diarrhoeic calves and clinically normal calves are shown in Tables 3.5 and 3.6 respectively, and summarised in Table 3.7.

(a) Rotavirus

Rotavirus was detected in 43.4% of faeces from diarrhoeic calves and in 13.6% of faeces from clinically normal calves. The virus was present in the faeces of calves from 10 of 14 farms (71%) affected with diarrhoea, and in these herds the majority of animals were first affected between 2 and 9 days of age (Figure 3.6). Rotavirus associated diarrhoea was particularly severe on farms B, H and I where calf mortality was high (Table 3.4).

Table 3.4 Calf mortality on farms studied in the survey

| Farm | Previous mortality report ^a | Mortality during this survey ^b |
|------|--|---|
| A | 15-75%, March-September | Severe |
| B | Severe | Severe |
| C | 21-93%, March-September | Severe |
| D | 50% | Severe |
| E | 50-100% | Severe |
| F | no information | Severe |
| G | no information | None |
| H | no information | Intermediate |
| I | no information | Intermediate |
| J | 50%, August | Intermediate |
| K | None | None |
| L | None | None |
| M | no information | None |
| N | no information | None |

a; Higgins (1981).

b; Snodgrass, D.R. and Sherwood, D (1981). Report on the calf mortality problems experienced on the cattle project farms. Tripoli, Libya.

Table 3.5 Enteropathogens detected in the faeces of diarrhoeic calves

| Farm | number of faecal samples tested | number of faecal samples positive for | | | | Age of affected calves (days) | No. positive/ No. tested by ZST |
|------|---------------------------------|---------------------------------------|----|------|----------------|-------------------------------|---------------------------------|
| | | RV | CV | ETEC | Crypto | | |
| A | 6 | 1 | 0 | 4 | 1 | 1-3 | 3/4 |
| B | 10 | 8 | 1 | 0 | 2 | 4-8 | 4/5 |
| C | 6 | 0 | 0 | 0 | 0 | 2 | 3/4 |
| D | 2 | 1 | 0 | 0 | 0 | 2-5 | 1/2 |
| E | 5 | 2 | 0 | 3 | 0 ^a | 1-3 | 1/3 |
| F | 2 | 0 | 0 | 0 | 0 | 3-5 | 1/3 |
| G | 3 | 1 | 0 | 0 | 0 | 4-5 | 2/2 |
| H | 6 | 2 | 1 | 0 | 0 | 1-13 | 1/3 |
| I | 4 | 4 | 0 | 0 | 1 | 4-7 | 3/4 |
| J | 3 | 0 | 0 | 0 | 2 | 2-3 | 5/5 |
| K | 2 | 0 | 0 | 0 | 0 | 40 | 1/2 |
| L | 1 | 1 | 0 | 0 | 0 | 31 | NT |
| M | 1 | 1 | 0 | 0 | 0 | 4 | NT |
| N | 2 | 2 | 0 | 0 | 0 | 4-8 | 2/3 |

a; only 3 calves sampled.

NT; no samples taken.

Crypto; Cryptosporidium sp.

RV; rotavirus.

CV; coronavirus.

Table 3.6 Enteropathogens detected in the faeces
of clinically normal calves

| Farm | number of faecal samples tested | number of faecal samples positive for | |
|------|--|--|--------------------|
| | | RV | CV, ETEC or Crypto |
| A | 1 | 0 | 0 |
| B | 2 | 2 | 0 |
| C | 2 | 0 | 0 |
| D | 3 | 0 | 0 |
| F | 2 | 0 | 0 |
| G | 3 | 0 | 0 |
| H | 2 | 0 | 0 |
| J | 2 | 0 | 0 |
| K | 3 | 0 | 0 |
| L | 1 | 0 | 0 |
| N | 1 | 1 | 0 |

RV; rotavirus.

CV; coronavirus.

Crypto; Cryptosporidium sp.

Table 3.7 Summary of the microbiological findings in the Libyan survey

| Calves | number of faecal samples tested | number of faecal samples positive for | | | |
|------------|--|---------------------------------------|-----------------------|---------------------------|-------------|
| | | rotavirus | ETEC | <u>Cryptosporidium</u> sp | Coronavirus |
| Normal | 22 | 3 (13.6) | 0 | 0 | 0 |
| Diarrhoeic | 53 | 23 ^a (43.4) | 7 ^b (13.2) | 6 ^c (11.7) | 2 (3.8) |





Percentage isolation in parentheses.

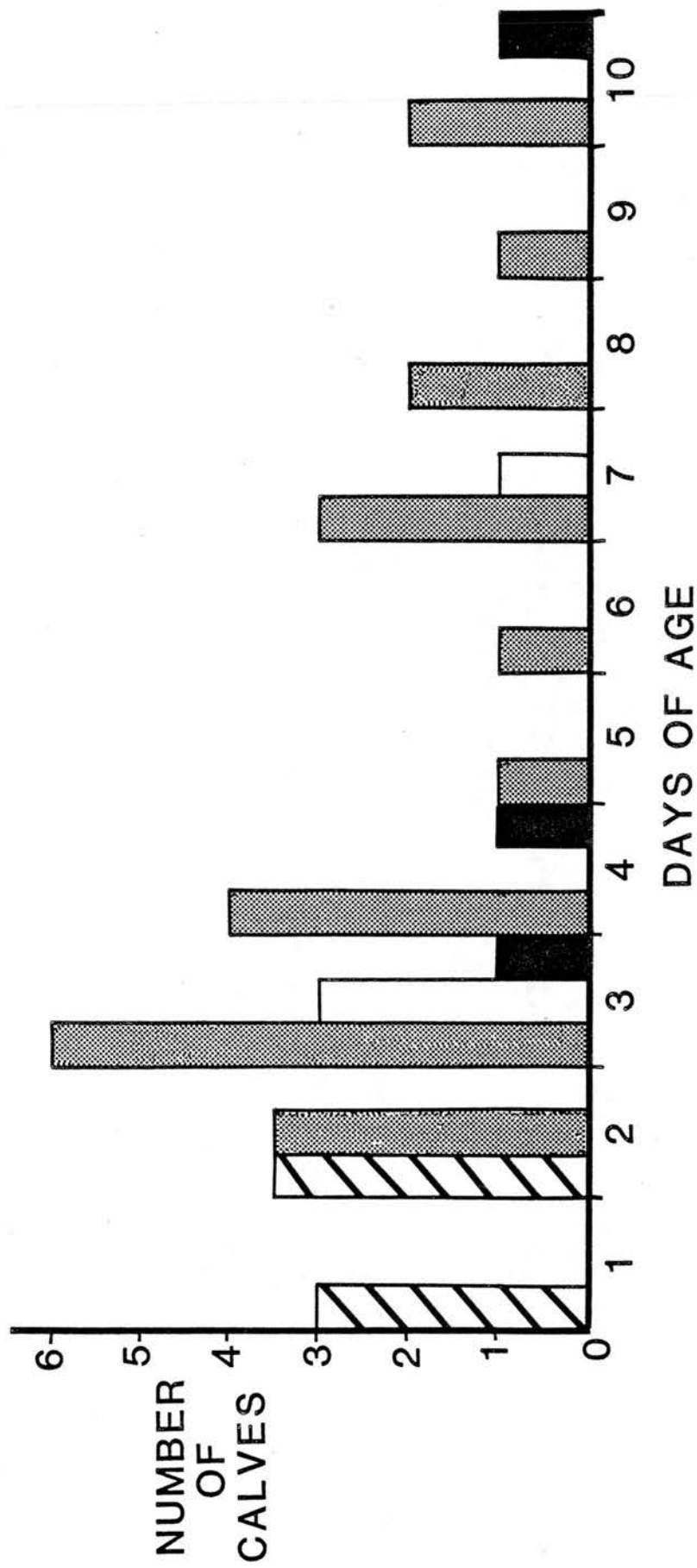
a; P = 0.07 by Chi square.

b; P = 0.17 by Chi square.

c; only 51 calves tested.

Figure 3.6 Age distribution of calves infected with enteropathogens in the Libyan survey.

| | |
|---------------------------|---|
| ETEC |  |
| rotavirus |  |
| <u>Cryptosporidium</u> sp |  |
| coronavirus |  |



(b) Coronavirus

Coronavirus was detected in the faeces of only 2 diarrhoeic calves out of the 53 diarrhoeic and 22 clinically normal calves examined. These calves were from farms B and H.

(c) Cryptosporidium sp

This organism was observed in the faeces of 6 diarrhoeic calves from farms B, I and J. The calves were 3 to 7 days of age when demonstrated to be infected (Figure 3.6), and showed intermittent diarrhoea. Cryptosporidium sp were not demonstrated in the faeces of any clinically normal calf.

(d) Salmonella spp

Three possible Salmonella spp isolations were made from single calves on separate farms. Confirmation and complete species identification of the isolates was not carried out.

(e) ETEC

These organisms were detected in diarrhoeic calves on farms A and E in which typical enterotoxigenic colibacillosis was observed in calves under 24 hours of age (Figure 3.6), and was associated with severe mortality (Table 3.4). The problem was particularly severe on farm A where most calves died within 2 to 3 days of the onset of diarrhoea. The non-haemolytic K99⁺ E. coli isolated from 7 calves were further characterised at Moredun Research Institute. All isolates produced STa but not VT or LT, and caused dilatation of calf gut loops (Table 3.8). There was complete correlation between STa and K99 antigen possession by isolates.

The 304 K99⁻ E. coli isolates obtained were not tested for STa in the infant mouse test or for their enterotoxigenicity in calf

Table 3.8 Characteristics of non-haemolytic K99⁺ E. coli isolated from diarrhoeic calves

| Farm/calf number | isolates positive/ isolates tested | | | Gut loop ^a (ml/cm) | Serotype | Antibiotic resistance ^b |
|------------------|---------------------------------------|-----|-----|----------------------------------|-----------------------------------|---------------------------------------|
| | STa | LT | VT | | | |
| A/4 | 1/1 | 0/1 | 0/2 | 1.5 | O?:K25:H ⁻ | S, Ct, N |
| A/5 | 2/2 | 0/2 | 0/2 | 3.29 | O?:K ⁻ :H ⁻ | S, Ct |
| A/6 | 1/1 | 0/1 | 0/1 | 2.00 | | S, Ct |
| A/7 | 3/3 | 0/3 | 0/2 | NT | | S, Ct |
| E/1 | 3/3 | 0/3 | 0/2 | 1.89 | O8:K85ab:H2 | S, Ct, C |
| E/4 | 3/3 | 0/3 | 0/3 | 2.36 | O8:K85ab:H2 | S, A |
| E/5 | NT | NT | NT | NT | | NT |

a; >1ml/cm is a positive reaction, only 1 isolate tested in at least 2 loops.

b; Isolates tested against antibiotics used in Chapter 3, Section A.

NT; not tested.

A; Ampicillin
C; Chloramphenicol
Ct; Chlortetracycline
N; Neomycin
S; Streptomycin

ligated gut loops as only K99⁺ isolates were brought back to Britain. The antibiotic resistance patterns of isolates from farm A were similar to each other, but different from the farm E isolates.

Serotyping indicated that K99⁺ E. coli from farm E were of the O8:K85ab:H2:K99 serotype, whereas those from farm A were of an unidentified serotype O?:K25:H⁻:K99.

Mixed infections

Six diarrhoeic calves had mixed enteric infections when sampled. Three, 2-day-old calves, one on farm A and two on farm E had concurrent ETEC and rotavirus infections. Two calves were infected with rotavirus and coronavirus (from farms B and H) and one calf from farm I had a rotavirus/Cryptosporidium sp infection.

ZST

Forty sera were tested, of which 27 (68%) were considered to contain satisfactory immunoglobulin concentrations. Of the 13 diarrhoeic calves examined from which an enteropathogen was detected, 4 had low immunoglobulin levels, whilst in the 14 diarrhoeic calves from which enteropathogens were not recovered, 8 calves had low immunoglobulin levels.

DISCUSSION

The disease picture and prevalence of enteropathogens in this survey were very similar to those described in Chapter 3, Section A. Rotavirus was the most commonly detected enteropathogen in both surveys, with coronavirus and Cryptosporidium sp being of minor importance. ETEC were found to be of similar prevalence in both surveys, but were isolated with rotavirus in 3 of 7 ETEC infected calves in the present study.

Severe calf mortality was observed on 6 farms (A to E and J). As reported by Higgins (1981), the majority of deaths could be attributed to dehydration resulting from acute diarrhoea. This was apparent on farms A and E where ETEC infections and on farms B, E, H and I where rotavirus infections were associated with severe calf mortality.

Enteropathogens were detected in 32 of 53 diarrhoeic calves (60.3%) and 3 of 22 clinically normal calves (13.6%). These figures show a similar pattern to the survey results from Scotland and Northern England (48.3% diarrhoeic and 25.0% clinically normal calves respectively). Thus, in both surveys an infectious cause could not be demonstrated in 40 to 50% of diarrhoea cases. Other surveys (Morin, Lariviere and Lallier, 1976; Moon, McClurkin, Isaacson, Skartvedt, Gillette and Baetz, 1978) have reported 25 to 40% of calf diarrhoea cases to be of undetermined aetiology. The sensitivity of the diagnostic tests or time of faecal sampling could have influenced the results. New, as yet undetected enteropathogens or management factors could also contribute to the diarrhoea syndrome.

In conclusion, the calf diarrhoea survey in Libya revealed a disease picture similar to that encountered in the Scotland and Northern England survey. This present survey together with surveys undertaken in other countries suggests that the infectious causes of calf diarrhoea are the same throughout the world. Vaccination of pregnant cows against enteropathogens, ensurance that calves receive colostrum, and an increase in the standards of hygiene may help to reduce the incidence and severity of calf diarrhoea in the Libyan Dairy Project.

CHAPTER 4 SECTION A

DEVELOPMENT OF AN ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) FOR THE ASSAY OF K99 ANTIGEN AND ANTIBODIES

INTRODUCTION

Over the past 10 years the ELISA system originally devised by Engvall and Perlmann (1972) has been developed to detect specific antigens in complex antigen mixtures, and as a means of detecting specific antibodies against particular antigens. Advantages of the system are that it is highly sensitive, provides efficient usage of antigens and antisera, and may be used for the rapid screening of many samples. However, the extreme sensitivity of the ELISA system necessitates the use of highly specific reagents to avoid the occurrence of non-specific cross-reactions.

The ELISA system described in this work was developed principally for two purposes, namely the detection of antibodies to K99 antigen both in the sera of experimental laboratory animals and in bovine sera and whey, and for the detection of K99⁺ E. coli in bovine faeces samples.

MATERIALS AND METHODS

Buffers and reagents

(i) Coating antibody diluent comprised carbonate/bicarbonate buffer (pH 9.6) which contained 0.05M Na₂CO₃, 0.05M NaHCO₃ and 0.004M NaN₃ in distilled water.

(ii) Plate washing solution (PBS/Tween) comprised 0.5g/l of polyethylene sorbitan monolaureate (Tween-20, Sigma Chemical Company, Poole, Dorset, England) in PBS (pH 7.2).

(iii) Substrate buffer comprised diethanolamine 100ml, distilled water 840ml, 0.005M $MgCl_2$ and 0.004M NaN_3 . The solution was adjusted to pH 9.6 with 1M HCl.

Coating antibody diluent and substrate buffer were stored at 4 °C and discarded after 28 days.

(iv) Substrate; p-nitrophenyl phosphate di-sodium, (Sigma) was prepared freshly for each test as a 1g/l solution in substrate buffer.

(v) Reaction arrester; 3M NaOH.

(vi) Microtitre plates; 96 well polystyrene plates (Type M129A, Dynatech Laboratories Ltd, Billingham, Sussex, England) were used.

Preparation of a crude K99 antigen extract

This extract, from E. coli strain B41, provided by Dr. L.K. Nagy (Wellcome Research Laboratories, Beckenham, Kent, England) was the same as described for the oil adjuvant vaccine in Chapter 4 Section B.

The protein concentration of the crude K99 antigen extract estimated by the Lowry method (Lowry, Rosenborough, Farr and Randall, 1951) was 2.16mg/ml. The antigen was tested against antiserum to strain EC46 and K99 antiserum obtained from Dr. W.H. Jansen (Bilthoven, Netherlands) by the immunodiffusion test (Figure 4.1) described in Chapter 2.

Preparation of specific K99 antiserum for use as ELISA coating antibody and conjugated antibody

K99 antiserum was prepared in rabbits by Dr. Jansen using purified K99 antigen obtained from an ultrasonic extract of E. coli

Figure 4.1 Immunodiffusion test to examine a crude K99 antigen extract.

K99; antigen extract prepared by Dr. L.K. Nagy
from E. coli strain B41 (O101:K-:K99).
aK99; K99 antiserum obtained from Dr. W.H. Jansen.
aEC46; antiserum prepared against whole organisms
of E. coli strain EC46 (O101:K?:K99).

Staining was by coomassie blue.

Note, the 2 lines obtained with the anti-EC46 serum suggests that there are antigens other than K99 antigen present in the K99 antigen extract.

65a

k99

a99

a46

K99

strain 0101:K(A)?:K99:NM by the preparative electrophoresis method (Guinee, Jansen and Agterberg, 1976).

The fractionation of this antiserum and all enzyme-IgG conjugation procedures were performed by Mr. A. McL. Dawson, Moredun Research Institute. The IgG fraction was isolated on a protein A column by the method of Goding (1976). This fraction, which contained 2.5mg/ml of protein, was stored in aliquots at -20°C . When required, thawed aliquots were diluted to 0.5mg/ml and conjugated to alkaline phosphatase (Sigma Type VII) by the method of Engvall and Perlmann (1972). The conjugated antibody was stored in aliquots at -20°C .
Preparation of conjugated antibody for the detection of bovine IgG, IgA and IgM

The IgG fractions of rabbit anti-bovine IgG, IgA and IgM which were obtained commercially (Miles Laboratories, Ellchart, Indiana, USA), were conjugated with alkaline phosphatase by the same technique.

General ELISA methods

(a) The K99 ELISA test

Microtitre plate wells were sensitised overnight at 4°C with 100 μl volumes of an appropriate dilution of coating antibody in coating antibody diluent. Plates were washed three times in PBS/Tween, then positive and negative antigen control samples and test samples diluted in PBS/Tween were added to duplicate wells in 100 μl volumes. The plates were incubated at 37°C for 3 hours then washed three times in PBS/Tween.

An appropriate dilution of rabbit anti-K99 alkaline phosphatase conjugate (in PBS/Tween) was added in 100 μl volumes to each well and

incubated at 37 °C for 1 hour. The plates were washed three times in PBS/Tween and 100µl of freshly made enzyme substrate was added to each well.

After incubation at room temperature for 1½ hours the reaction was stopped by the addition of 15µl of 3M NaOH. The optical density was read in an automated scanner ('Titertek Multiskan', Flow Laboratories) at a wavelength of 405nm.

(b) The blocking ELISA test for detection of specific K99 antibodies in sera from rabbits, rats, guinea pigs and mice

The technique was essentially the same as for the K99 ELISA test with the following modifications: After washing off excess coating antibody, K99 extract diluted in PBS/Tween was added to each well. The plates were incubated at 37 °C for 3 hours and washed 3 times in PBS/Tween. Serial two-fold dilutions of test and positive and negative control sera in PBS/Tween were added to wells in 100µl volumes and the plates incubated for 1 hour at 37 °C. Conjugate and enzyme substrate was then added as described in the previous section.

The blocking titre of a serum was defined as the reciprocal of that dilution which reduced by 50% the optical density at 405nm of the unblocked positive antigen control.

(c) The sandwich ELISA to detect bovine K99 antibodies in sera and whey

The test was essentially the same as the K99 ELISA test with the following modifications: Diluted K99 antigen extract was added to each antibody coated well in 100µl volumes, incubated at 37 °C for 3 hours, then excess antigen was washed off and 100µl of bovine sera or whey diluted 1/100 and 1/1000 in PBS/Tween were added to duplicate wells. A standard bovine K99 antiserum was included on

each plate in doubling dilutions between 1/200 and 1/256000.

Rabbit anti-bovine IgG conjugated with alkaline phosphatase was used. Plates were incubated with the enzyme substrate for 2 hours at room temperature.

The K99 antibody titre of test samples was derived from a calibration curve produced by 8 dilutions of the standard serum. The standard serum titre was defined as the reciprocal of that dilution which gave an optical density (OD_{405}) of 0.1. An OD_{405} of less than 0.1 was regarded as negative, since sera known to be devoid of K99 antibodies gave variable values below this point, and test samples diluted 1/100 which gave an OD_{405} below 0.1 were assigned a titre of <100.

That dilution of the standard serum which would give an OD_{405} identical to any given test sample (the standard equivalent dilution, SED) was calculated by regression analysis of the standard serum dilution (as \log_{10}) plotted against the OD_{405} (as \log_{10}) (Figure 4.2) using the following equation:

$$\log_{10} SED = \frac{\log_{10} OD_{405} \text{ of test sample at } 1/100 \text{ or } 1/1000 \text{ dilution} - A}{B}$$

where A = intercept of standard line on the y-axis (Figure 4.2) and

B = gradient of standard line.

The titre of the test sample was then given by;

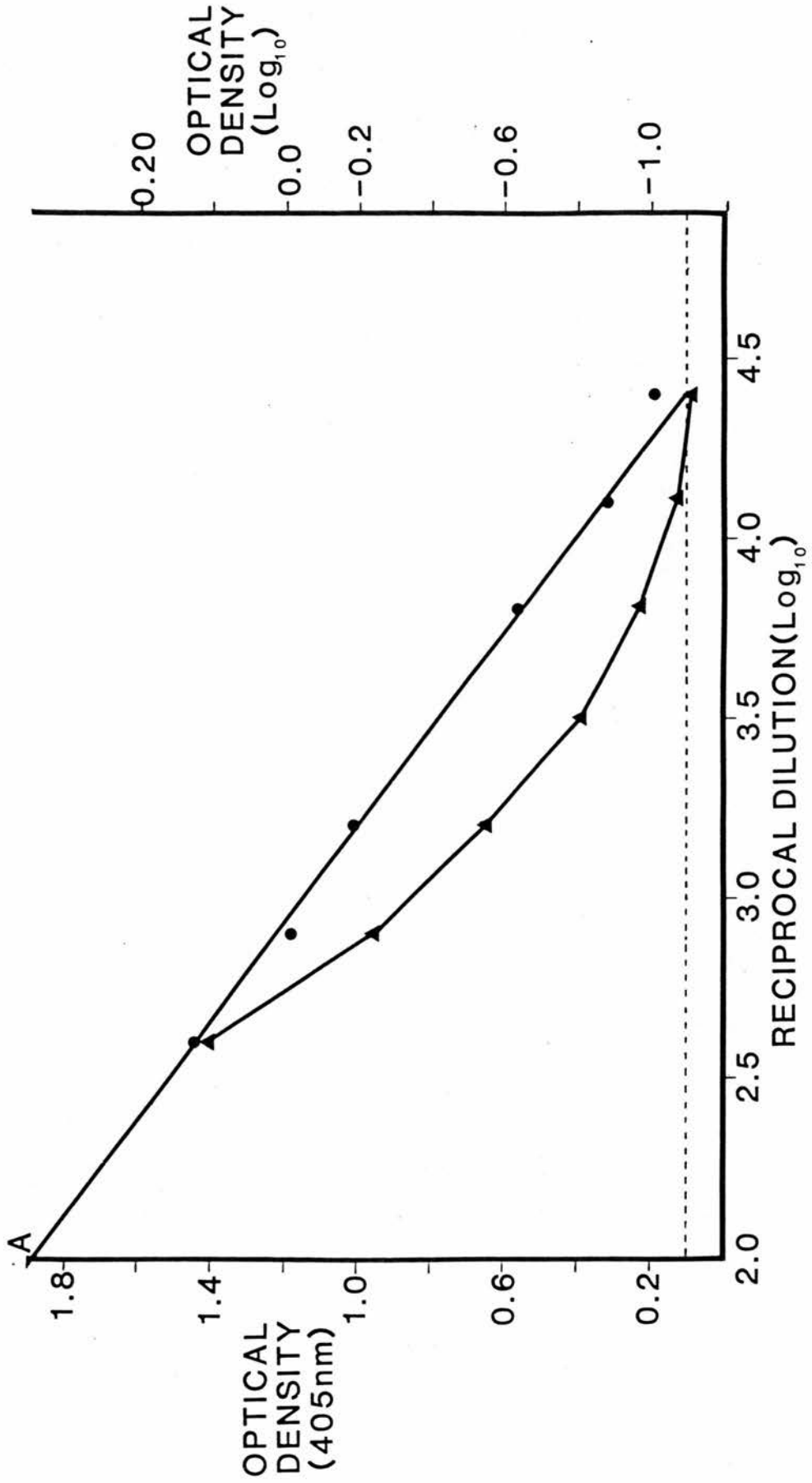
$$\frac{\text{Reciprocal of dilution of test sample (100 or 1000)} \times \text{titre of standard serum}}{SED}$$

(d) Assay of the optimal dilutions of coating antibody, conjugated antibody and crude K99 antigen extract for use in the ELISA

Five microtitre plates were sensitised with doubling dilutions of

Figure 4.2 Optical density (at 405nm) of dilutions of standard bovine K99 antiserum in the sandwich ELISA.

▲ — ▲ OD_{405} versus \log_{10} reciprocal dilution.
● — ● $\log_{10} OD_{405}$ versus \log_{10} reciprocal dilution.
A; intercept of standard line on the y-axis.



coating antibody from 1/250 to 1/32000. Each of the 5 plates was coated with a dilution (1 in 25, 50, 100, 200 or 400) of crude K99 antigen extract. Anti-K99 conjugate dilutions from 1/20 to 1/2560 were then applied to every plate in a checker-board pattern relative to the coating antibody dilutions.

The optimal dilutions of the reagents were regarded as the maximum dilutions which gave an OD_{405} of 1.0 after $1\frac{1}{2}$ hours incubation with substrate at room temperature. These working dilutions were, 1/4000 for coating antibody; 1/100 for crude K99 antigen and 1/160 for conjugated K99 antibody.

The conjugated rabbit anti-bovine IgG was titrated using coating antibody at 1/4000 and crude K99 antigen at 1/100. The optimal dilution of the conjugate was taken to be that which gave an OD_{405} of less than 0.1 with negative sera and of 1.5 with standard bovine K99 antiserum diluted 1/400. This working dilution was 1/100.

EXPERIMENT 1: SPECIFICITY OF THE ELISA FOR K99 ANTIGEN

METHODS

K99⁺, K99⁻ and F41 strains of E. coli (Table 4.1) were grown overnight at 37 °C and at room temperature on minca-Isovitalex agar. K88⁺ and 987P⁺ strains of E. coli were similarly grown on 5% blood agar. Suspensions were made in PBS to give approximately 10^9 organisms/ml (Brown Opacity Tube No. 2). Each suspension was applied in 100µl volumes to 4 wells of a sensitised microtitre plate. Two wells were blocked with antiserum (diluted 1/20) raised to E. coli strain K12:K99 and adsorbed with a K12 strain. An $OD_{405} > 0.1$ was considered positive and an $OD_{405} < 0.1$ was considered negative for the test. Blocking was regarded as successful if the OD_{405} was reduced by >50%.

Table 4.1 Assessment of the specificity of the ELISA for K99 antigen

| Strains | OD ₄₀₅ after cultivation at | | OD ₄₀₅ after blocking organisms cultivated at 37 °C |
|---|--|---------------------|--|
| | 37 °C | Room temperature | |
| 09 ⁺ :K30 ⁺ :K99 ⁺ | >2.00 | 0.00 | 0.60 |
| 09 ⁺ :K30 ⁺ :K99 ⁻ | 0.00 | NT | NT |
| 09 ⁺ :K30 ⁻ :K99 ⁺ | >2.00 | NT | 0.55 |
| 09 ⁻ :K30 ⁻ :K99 ⁺ | >2.00 | NT | 0.87 |
| 09 ⁺ :K30 ⁻ :K99 ⁻ | 0.00 | NT | NT |
| K12 ^a | 0.00 | NT | NT |
| K12:K99 ^a | 1.33 | 0.00 | 0.45 |
| K12:K88 ^a | 0.00 | NT | NT |
| 987P ^a | 0.00 | NT | NT |
| F41 ^b | 0.00 | NT | NT |
| B41 | 1.40 | 0.00 | 0.54 |
| EC6 | 0.70 | 0.00 | 0.32 |
| EC7 | 0.01 | NT | 0.00 |
| bovine rotavirus | 0.00 ^c | NT | 0.00 |
| bovine coronavirus | 0.00 ^d | NT | 0.00 |

a; organisms grown on 5% sheep blood agar.

b; mutant derived from strain B41, lacks K99 antigen, but possessed the F41 antigen (supplied by N. Chanter, Compton, England).

c; tissue culture and faeces.

d; tracheal organ culture fluid and faeces.

The K99 antigen ELISA was checked for non-specific cross reactions with 2 bovine enteric viruses isolated at the Moredun Research Institute. A coronavirus propagated in calf tracheal organ culture with a haemagglutination (HA) titre of 8, and a rotavirus grown in bovine embryonic kidney cells (which were subsequently freeze-thawed 3 times to release virus) with a titre of 10^5 - $10^{6.5}$ TCID₅₀/ml were used. In addition faeces which contained, separately, coronavirus at a titre of 256 in the haemadsorption-elution-haemagglutination test, and rotavirus of unknown titre were tested.

RESULTS

Results of the specificity assessment of the ELISA for K99 antigen are shown in Table 4.1.

All K99⁺ strains grown at 37 °C gave positive reactions in the ELISA and were specifically blocked by adsorbed anti-K12:K99 serum. The K99⁻ strains grown at 37 °C or K99⁺ strains grown at room temperature were negative in the test.

There were no cross-reactions with other pilus antigens such as K88, 987P or F41, nor with cell cultures or faecal suspensions containing the coronavirus and rotavirus.

EXPERIMENT 2: SENSITIVITY OF THE ELISA FOR THE DETECTION OF K99 ANTIGEN IN EXTRACTS FROM K99⁺ E. COLI AND ON WHOLE ORGANISMS

METHODS

Doubling dilutions of the K99 antigen extract described earlier, (Chapter 2) and of the crude K99 antigen extract supplied by Dr. L.K. Nagy were prepared. These contained 44µg and 2.16mg of protein per ml respectively before dilution.

Serial 10-fold dilutions of 5 K99⁺ E. coli strains grown overnight on minca-Isovitalex at 37 °C and suspended in PBS were used to determine the lowest number of whole organisms which would give an OD₄₀₅ in excess of 0.1

RESULTS

The lowest concentration of both extracts which gave a positive result was approximately 0.17µg of protein/ml.

Whole K99⁺ E. coli were detected at a minimum level of 2.5 to 10 x 10⁶ colony forming units (cfu)/0.1ml (Table 4.2).

EXPERIMENT 3: THE PRODUCTION OF K99 ANTIGEN BY K99⁺ E. COLI

DURING CULTURE

METHODS

Eighteen-hour cultures at 37 °C of E. coli strains EC6, B44, B41 and K12:K99 were used in 100µl volumes to inoculate 50ml of pre-warmed TSB. The cultures were grown at 37 °C on a rotary shaker. Viable counts were made on samples of broth taken every hour for 9 hours. The K99 content of each sample was tested by ELISA after storage at -20 °C.

RESULTS

The viable counts and K99 production over 9 hours by strains EC6, B44, B41 and K12:K99 are shown in Figure 4.3.

The peak K99 expression time occurred for strains B41 and EC6 after 4 hours during the exponential growth period whereas maximum expression of K99 antigen by strain K12:K99 was later and occurred after 6 hours. The organisms continued to increase in numbers after the peak K99 expression time, indicating a decrease in amount of K99 antigen expressed per organism.

Table 4.2 Sensitivity of the ELISA for the detection of
K99 antigen in extracts and in whole organism
suspensions

| Extract or organism suspension | Lowest concentration or titre at which OD ₄₀₅ >0.1 |
|---------------------------------------|--|
| Crude K99 antigen extract | 0.169 ^a |
| K99 antigen extract from Chapter 2 | 0.172 ^a |
| EC6 | 5 x 10 ⁶ ^b |
| B41 | 2.5 x 10 ⁶ ^b |
| B44 | 2.5 x 10 ⁶ ^b |
| K12:K99 ^c | 10 x 10 ⁶ ^b |
| K12 ^c | No reaction |

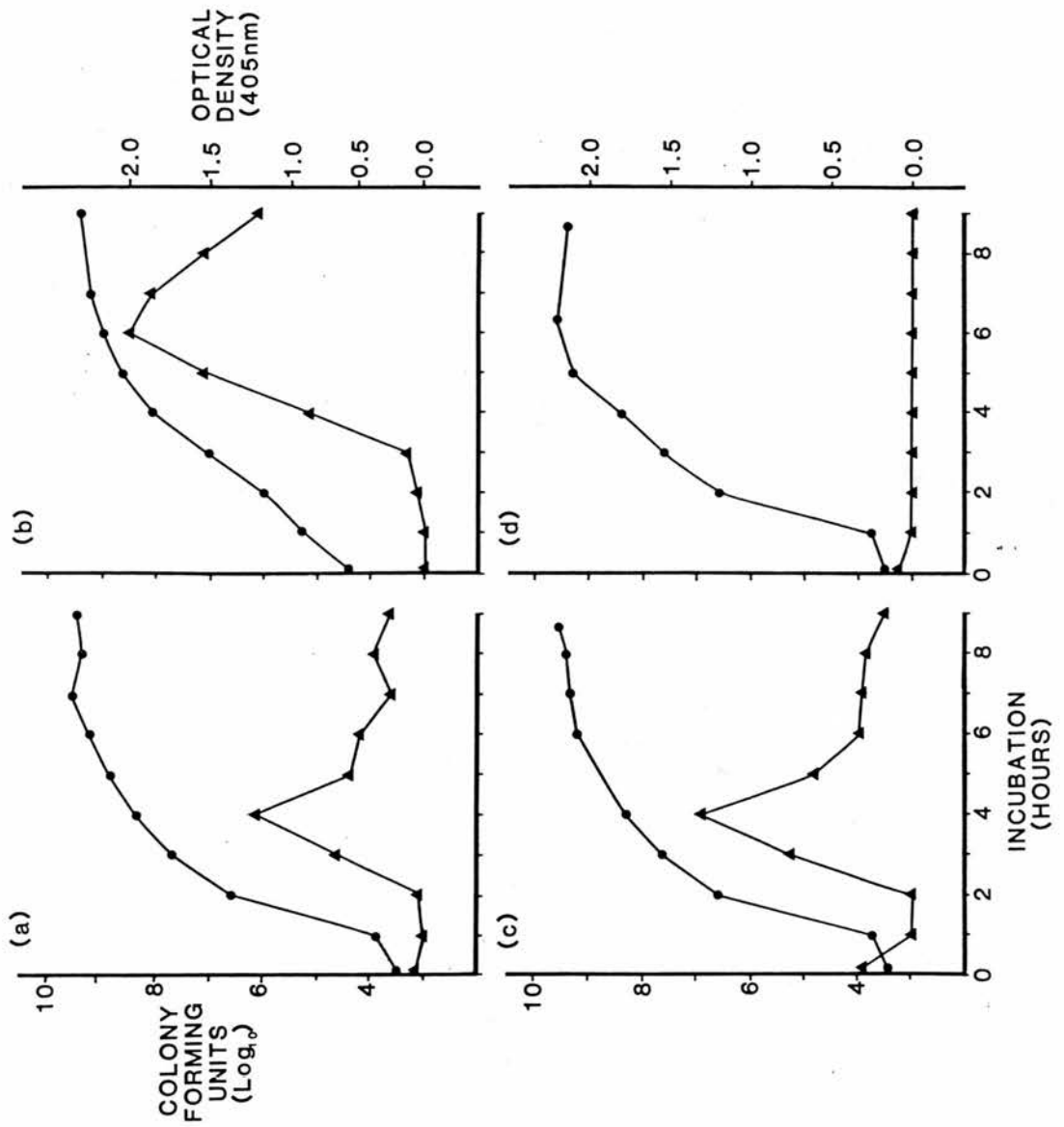
a; µg of protein/ml.

b; cfu/0.1ml.

c; grown on 5% sheep blood agar.

Figure 4.3 Amount of K99 antigen produced by 4 E. coli isolates grown in vitro as determined by the ELISA.

- (a) B41
 - (b) K12:K99
 - (c) EC6
 - (d) B44
- — ● cfu/ml
- ▲ — ▲ ELISA OD₄₀₅



K99 antigen was not detected at any time in the culture of strain B44.

EXPERIMENT 4: DETECTION OF K99⁺ E. COLI IN FAECES; A COMPARISON OF THE ELISA AND THE MINCA-ISOVITALEX ISOLATION/SLIDE AGGLUTINATION TECHNIQUE

MATERIALS AND METHODS

Faeces samples

Faeces were collected from 24 conventional calves over the first 6 days of life after challenge with E. coli strain B44 at under 18 hours of age. These calves were from vaccinated or unvaccinated cows used in Experiment 4 of Chapter 4, Section B.

K99⁺ E. coli screening

E. coli isolates grown on minca-Isovitalex agar were identified by the slide agglutination test with anti-K12:K99 serum adsorbed with the K12 E. coli strain.

In the ELISA, faeces diluted 1:4 in PBS/Tween were examined for the presence of K99 antigen after treatment for 45 seconds at room temperature in a sonicator water bath (Engisonic). Each faecal sample was dispensed into 4 wells; 2 wells were tested for K99 antigen as previously described, and two in which specific anti-K99 conjugate was not added acted as controls. This allowed non-specific colour changes of the substrate produced by non-specifically attached faecal products to be excluded.

The test was considered as not interpretable if colour changes in both pairs of wells were the same, since the colour change may have masked a positive result.

RESULTS

Of the 98 faecal samples tested by each technique, 48 were found to be negative and 42 positive by both tests (Table 4.3). Three samples were positive by culture on minca-Isovitalex only, and 5 by ELISA only. Thus the proportion of samples positive by ELISA and by minca-Isovitalex isolation slide agglutination technique were 48% and 46% respectively. The two techniques therefore have a comparable sensitivity.

EXPERIMENT 5: SPECIFICITY OF THE RABBIT ANTI-BOVINE IgG, IgM and IgA ALKALINE PHOSPHATASE CONJUGATES IN THE SANDWICH ELISA

MATERIALS AND METHODS

Wheys

Wheys were prepared from pooled colostrum and day 7 milk samples taken from a group of 6 vaccinated and 6 unvaccinated cows used in Experiment 4 of Chapter 4, Section B (Table 4.4).

Fractionation of wheys and testing of fractions in the ELISA

Fractionation of wheys was carried out by Mr. A. McL. Dawson on a 2.6cm x 81cm S300 column (Sephacryl, Pharmacia, Hounslow, England) eluted with 0.1M Tris and 1M NaCl pH 8.0. Samples were loaded in 2ml volumes after overnight dialysis at 4 °C in the column buffer. The column flow rate was 20.4ml/hour and 7ml fractions were collected and stored at -20 °C until tested for IgG, IgM and IgA K99 antibodies as described previously. Fractions were tested undiluted in duplicate.

Mancini Tests

Fractions from day 1 and day 7 whey from vaccinated cows were tested for IgG, IgA and IgM by the Mancini technique (see Chapter 2).

Table 4.3 Comparison of the ELISA test with minca-Isovitalex isolation/slide agglutination technique for the detection of K99⁺ E. coli in faeces

| Number of faeces samples | Result of test by | |
|-----------------------------|-------------------------------|-------|
| | Minca-Isovitalex isolation | ELISA |
| 42 | + | + |
| 3 | + | - |
| 5 | - | + |
| 48 | - | - |

++; positive test; $OD_{405} > 0.1$ in the ELISA, at least 1 of 5 colonies K99⁺ by slide agglutination.

-; negative test; $OD_{405} < 0.1$ in the ELISA, no K99⁺ colonies detected (5 tested) by slide agglutination.

Table 4.4 Origin of pooled whey samples used for the fractionation of bovine IgG, IgA and IgM (fractions used to check the specificity of the rabbit anti-bovine IgG, IgA and IgM alkaline phosphatase conjugates in the sandwich ELISA)

| Vaccination status of donor group ^a | Day of milk collection post parturition | Protein concentration of pooled whey sample (mg/ml) |
|---|--|---|
| Unvaccinated | 1 | 126.00 |
| | 7 | 12.70 |
| Vaccinated with oil adjuvant K99/ rotavirus vaccine | 1 | 119.00 |
| | 7 | 25.60 |

a; 6 cows per group.

RESULTS

No cross reactions were observed between the anti-bovine IgG conjugate and fractionated day 1 whey from unvaccinated cows, (Figure 4.4), whereas three distinct antibody peaks were seen which coincided with the IgM, IgA and IgG rich regions of day 1 whey from vaccinated cows. The anti-bovine IgA and IgM conjugates gave specific reactions in their respective class rich fractions (not shown in Figure 4.4) but both cross reacted in the immunoglobulin rich areas of the other 2 classes.

The reactivity of the three conjugates with fractions of day 7 whey is shown in Figure 4.5. The anti-IgA and anti-IgM conjugates reacted strongly in the sandwich ELISA test in fractions which, by the Mancini test contained only IgG.

EXPERIMENT 6: THE BLOCKING EFFECT OF FRACTIONATED WHEY IN THE ELISA

METHODS

All 4 fractionated wheys used in Experiment 5 were examined for their blocking activity in the ELISA. Each fraction was applied undiluted to duplicate wells in 100µl volumes after the plates had been sensitised with K99 antigen. The methodology has been described previously.

RESULTS

The results are shown in Figures 4.6 and 4.7. Specific blocking activity was apparent with day 1 and day 7 fractionated whey from vaccinated but not from unvaccinated cows. Considerable blocking was produced by all immunoglobulin rich fractions, irrespective of the class in day 1 whey from vaccinated cows (Figure 4.6). The

Figure 4.4 Immunodiffusion (Mancini) for IgG, A and M and ELISA test for K99 antibodies (using rabbit anti-bovine IgG alkaline phosphatase conjugate) in Day 1 fractionated whey from vaccinated and unvaccinated cows.

▲ —▲ Whey from unvaccinated cows.
■ —■ Whey from vaccinated cows.

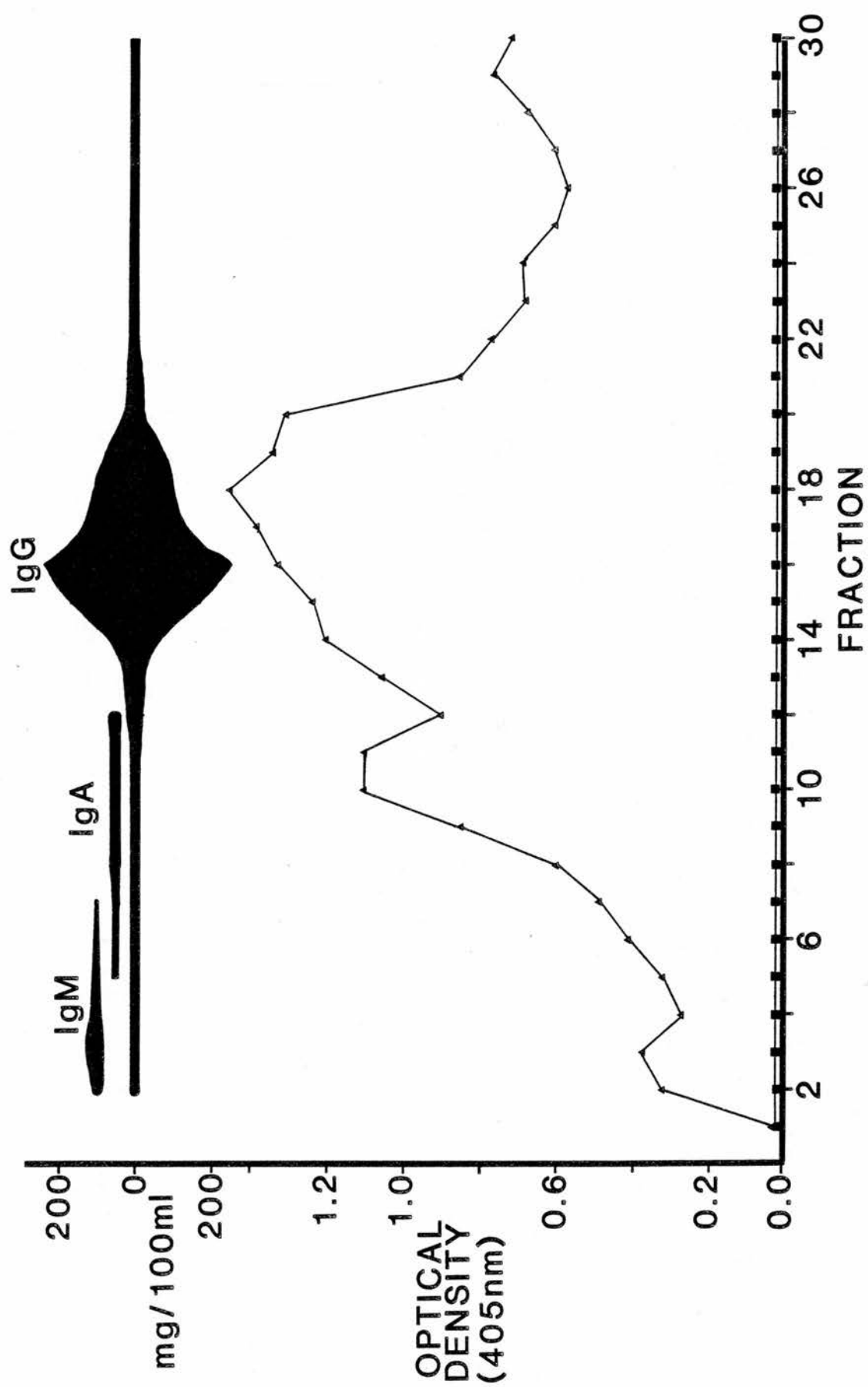


Figure 4.5 Immunodiffusion (Mancini) for IgG, A and M and ELISA test for K99 antibodies (using rabbit anti-bovine IgG, A and M alkaline phosphatase conjugates) on Day 7 fractionated whey from vaccinated cows.

- ▲ — ▲ anti-bovine IgG conjugate.
- — ■ anti-bovine IgM conjugate.
- — ● anti-bovine IgA conjugate.

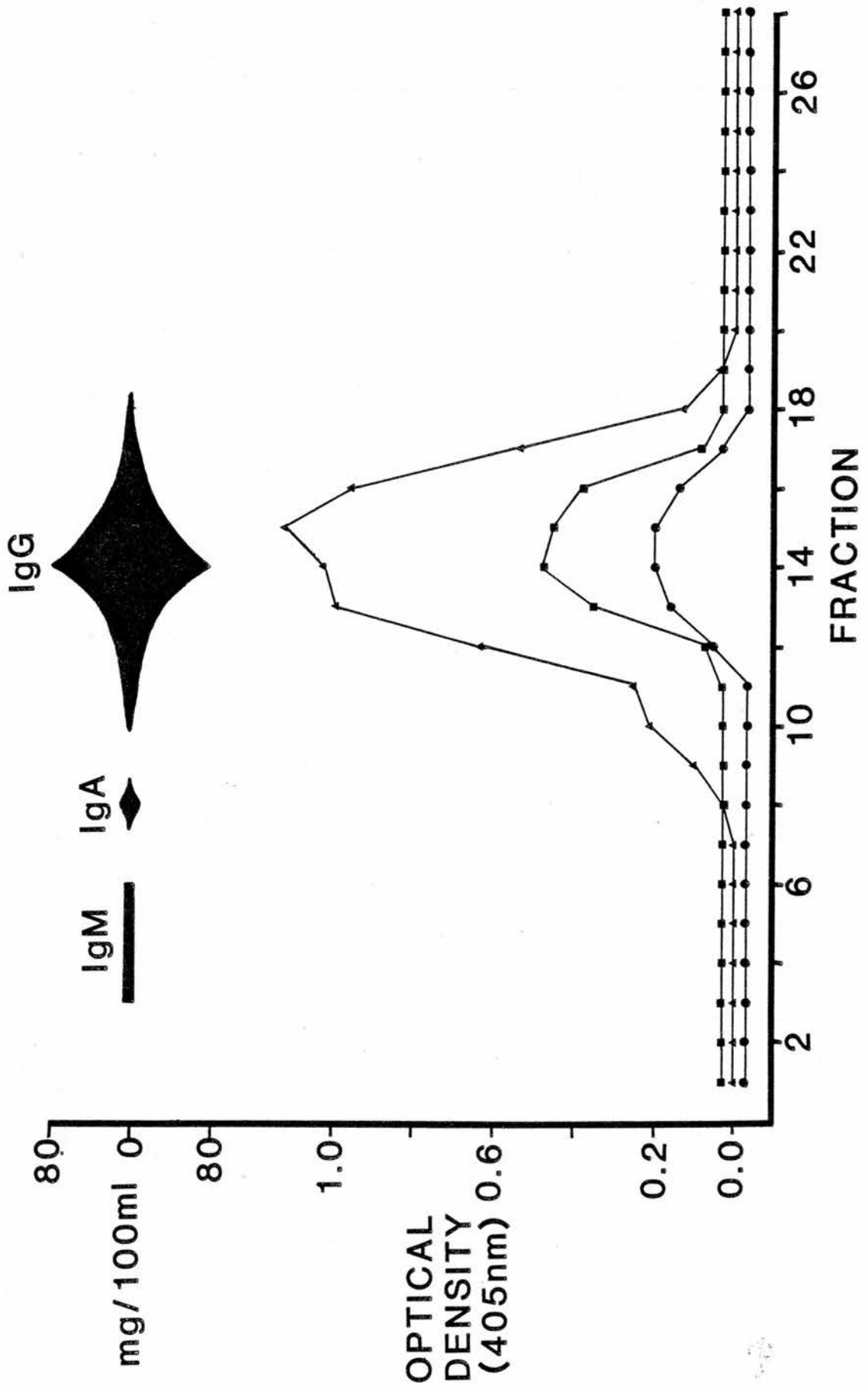


Figure 4.6 The blocking ELISA of fractionated Day 1 whey from vaccinated and unvaccinated cows.

▲ — ▲ whey from unvaccinated cows.
■ — ■ whey from vaccinated cows.

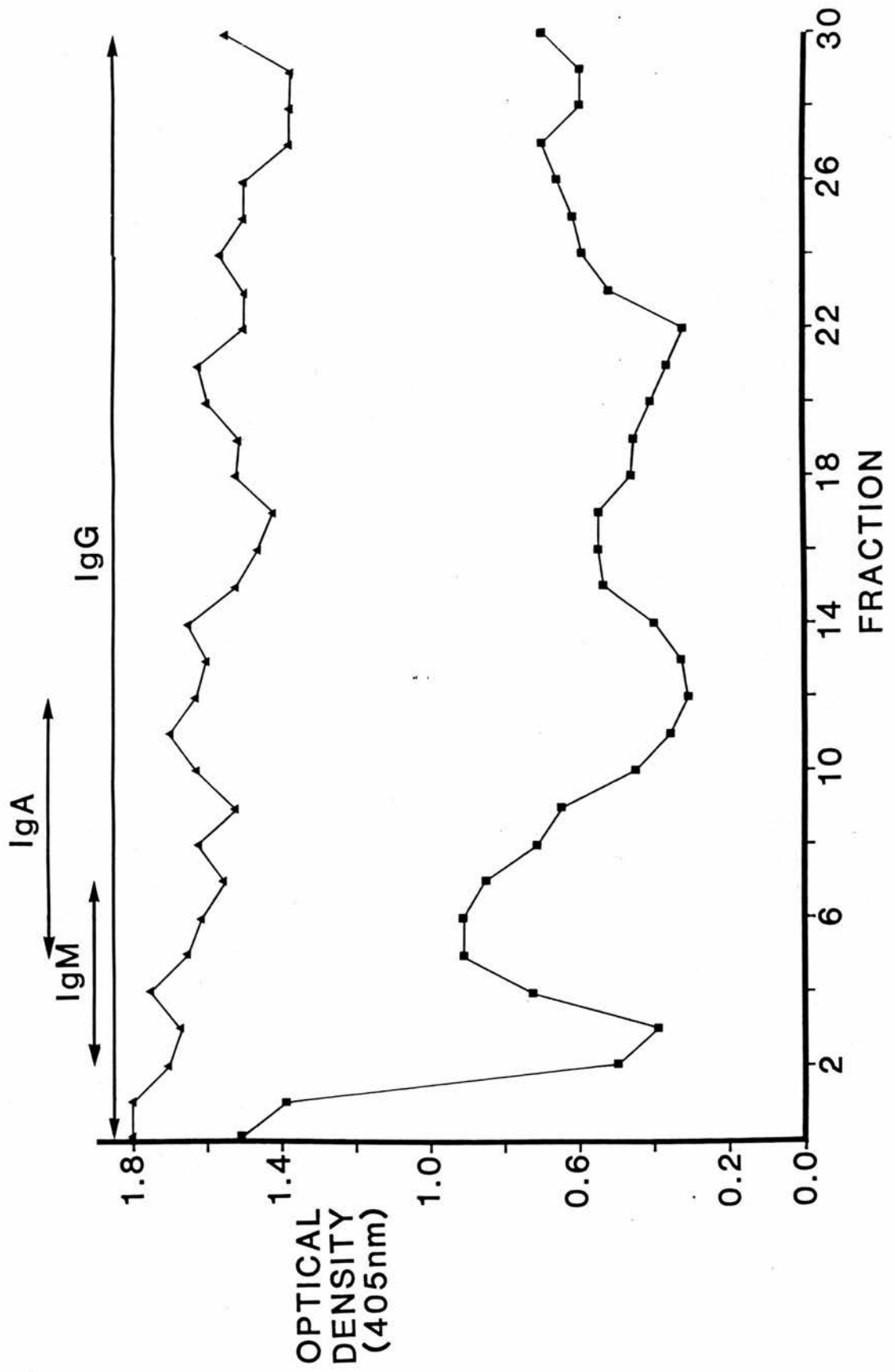
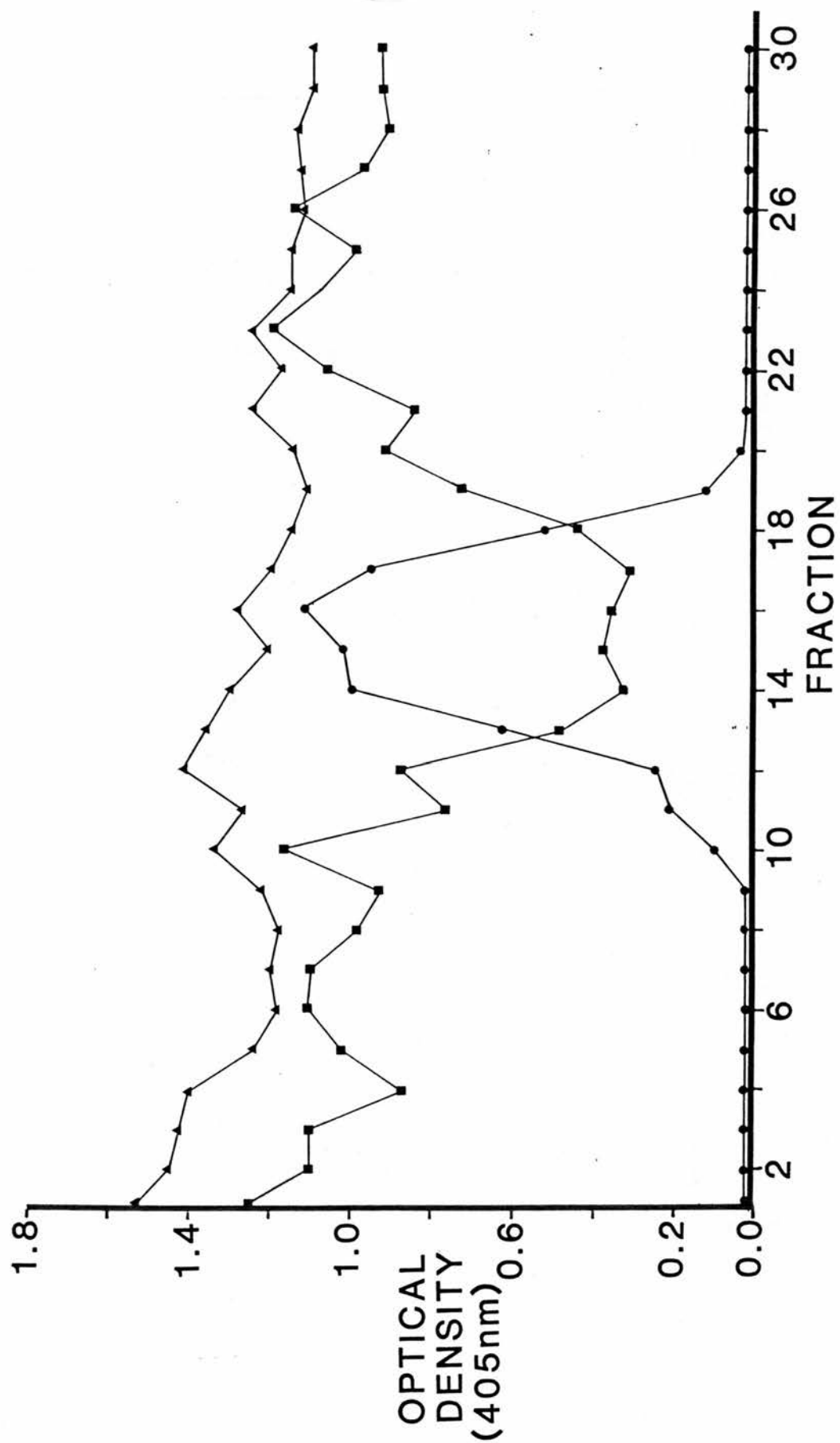


Figure 4.7 The blocking ELISA compared with the sandwich ELISA of fractionated Day 7 whey from vaccinated and unvaccinated cows.

- ▲ — ▲ whey from unvaccinated cows.
- — ■ whey from vaccinated cows.
- — ● sandwich ELISA titres of Day 7 whey from vaccinated cows.

74d



blocking activity of day 7 fractionated whey from vaccinated cows was confined to the IgG rich region (Figure 4.7).

EXPERIMENT 7: TITRATION OF RABBIT ANTISERA TO DIFFERENT E. COLI STRAINS, AND OF SERUM FROM A GNOTOBIOTIC LAMB IMMUNISED WITH A K99 ANTIGEN EXTRACT

METHODS

The blocking ELISA was used to assay antisera prepared in rabbits (Chapter 2) with whole organisms of 4 E. coli strains namely, EC6, EC46, B44 and K12:K99. The blocking ELISA titre and sandwich ELISA titre of a gnotobiotic lamb antiserum against a K99 antigen extract prepared from strain B41 were assayed (Chapter 2). The rabbit anti-K99 serum donated by Dr. W.H. Jansen was also titrated by blocking ELISA.

RESULTS

The blocking titres of the various rabbit and lamb antisera are shown in Table 4.5. Titres of rabbit antisera to whole organisms ranged from 1500 to 2800. The lamb antiserum gave a blocking titre of >1280 and sandwich ELISA titre of 6400. The rabbit antiserum donated by Dr. W.H. Jansen had a blocking titre of 1800.

Two examples are given of the titration of antisera raised against strains EC6 and K12:K99 in the blocking ELISA (Figure 4.8). The 50% blocking titres of the two antisera can be calculated at an OD₄₀₅ of 1.0.

DISCUSSION

The ELISA has proved to be a very versatile and sensitive technique for the detection of K99 antigen and antibodies. The

Table 4.5 ELISA titres of antisera against various whole organisms and K99 extracts prepared in rabbits and a lamb

| Species of animal immunised | Strain of <u>E. coli</u> | Form of immunising antigen | Average ^a 50% blocking ELISA titre |
|-----------------------------|--------------------------|----------------------------|---|
| Rabbit | EC6 | W.O. | 1500 |
| " | EC46 | W.O. | 2400 |
| " | B44 | W.O. | 2100 |
| " | K12:K99 | W.O. | 2800 |
| " | B41 ^b | E | 1800 |
| " | - | - | No titre |
| lamb | B41 | E | >1280 (6400) ^c |

W.O.; whole organism.

E; extract.

a; antisera to whole organisms were prepared in 2 rabbits for each strain, the sera of which were pooled.

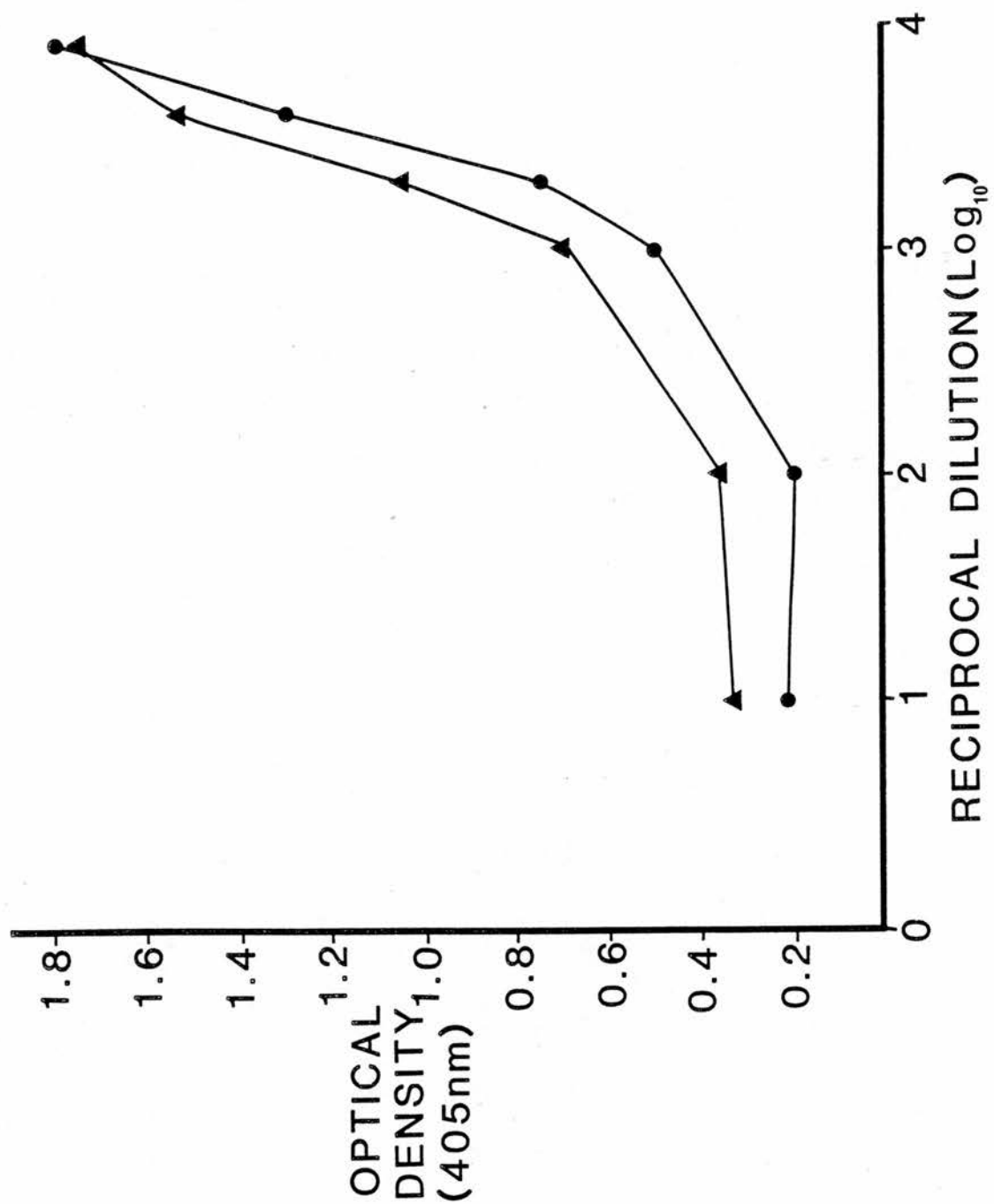
b; antisera donated by Dr. W.H. Jansen.

c; sandwich ELISA titre.

Figure 4.8 Blocking ELISA test of two rabbit antisera raised
to K99⁺ E. coli.

▲——▲ rabbit antiserum to strain EC6.

●——● rabbit antiserum to strain K12:K99.



methodology of the test described here was adapted from an ELISA test for the detection of rotavirus (Ellens and de Leeuw, 1977) but alkaline phosphatase instead of peroxidase was used in the present ELISA. During the development of the present ELISA, Ellens, de Leeuw and Rozemond (1979) reported the results obtained with a similar K99 ELISA using the same anti-K99 serum used in this Chapter. Ellens et al (1979) found the K99 ELISA to have low activity to bovine rotavirus and coronavirus, contrary to the negative results obtained in this Chapter with both viruses in either cultures or positive faeces samples.

An important factor in the development of a reliable K99 ELISA was the production of specific coating antibody for the detection of antigen. The results of Experiment 1 appeared to indicate that this had been achieved. K99⁺ E. coli grown at 37 °C gave positive reactions whereas the same strains grown at room temperature, or K99⁻ strains grown at 37 °C gave negative reactions. None of the other three pilus antigens, K88, 987P or F41 were detected.

The K99 extract used to produce the specific antiserum (obtained from Dr. W.H. Jansen) was derived from strain B41 and purified by electrophoresis (Guinee, Jansen and Agterberg, 1976) in which the K99 migrates to the cathode. F41 antigen under these conditions migrates to the anode (Morris, Thorns and Sojka, 1980; Morris, Thorns, Scott, Sojka and Wells, 1982). Thus complete separation of the two antigens would have occurred, and as expected F41 was not detected in this ELISA. The ELISA did not cross-react with non-pilus antigens on strain B41, as B41 grown at room temperature did not react in the ELISA.

The lowest levels of whole K99⁺ E. coli detected by the ELISA used in this study were $2.5 - 10 \times 10^6$ cfu/0.1ml, a range some 5 to 10 times higher than the minimal detection levels obtained by Ellens, de Leeuw and Rozemond (1978). The K99 antigen extracts tested (Table 4.2) were positive by the ELISA of this study at protein concentrations of approximately 0.17µg/ml. Since these extracts probably contained proteinaceous material other than K99, the lowest level of detectability of K99 protein was probably considerably less than this figure.

The maximum K99 expression times of the 4 K99⁺ E. coli strains tested varied from 4 to 6 hours post inoculation of broth. These peak expression times were much later than the 2 hours reported for strain K12:K99 by Issacson (1980), who used an enzyme linked antibody centrifuge assay. Both studies show that the maximum K99 antigen expression occurs during the logarithmic growth phase and rapidly declines in early or middle stationary phase. This decline could be due to a build up of metabolic products which may inhibit K99 antigen production as reported for L-alanine (de Graaf, Klaasen-Boor and van Hees, 1980) and glucose (Issacson, 1980). It is also possible that cell surface substances such as polysaccharides may mask K99 in some way. These findings on in vitro K99 expression, suggest that experimental animals should be challenged with 4 to 6 hour cultures of organisms in TSB to ensure maximum concentration of K99 antigen at the time of inoculation. K99 antigen expression by strain B44 was not detected in this study, possibly due to the mucoid nature of this strain causing a masking of the K99 antigen or a repression of its synthesis.

Similar results were obtained when the ELISA was compared with the minca-Isovitalex isolation/slide agglutination technique for the detection of K99⁺ E. coli in calf faeces (Table 4.3). These results are comparable to those recorded by Ellens, de Leeuw and Rozemond (1978). The advantages of the ELISA over the minca-Isovitalex method are that many faecal samples can be rapidly screened in one test, also, samples may be stored at -20 °C until convenient. The ELISA is quantitative for K99 antigen present in faecal samples and does not rely on chance isolation of a K99⁺ E. coli (Ellens et al, 1979). A disadvantage of the ELISA is that as E. coli isolates were examined for the presence of STa and LT, bacterial isolation had to be performed. For this reason the ELISA was not used for routine examination of faeces in this thesis particularly in Chapter 3.

It is important to emphasise the need for controls when faeces are examined for K99⁺ E. coli by the ELISA. The result of faecal products causing non-specific colour changes of the substrate can be eliminated using controls described in Experiment 4. A blocking stage using specific K99 antiserum and normal serum should be included to confirm the positive reaction in the unblocked test.

Conjugates made from the 3 commercially obtained rabbit anti-bovine IgG, IgA and IgM sera seemed to react in a non-specific manner in the sandwich ELISA as shown in Figures 4.4 and 4.5. Figure 4.4 indicates that the anti-bovine IgG conjugate may be cross-reacting with IgA and IgM class antibodies when tested against fractionated day 1 whey from vaccinated cows. Also the anti-IgA and IgM conjugates as well as reacting specifically, reacted non-specifically in the other two immunoglobulin-rich regions. The

anti-bovine IgA and IgM conjugates reacted most strongly in the sandwich ELISA test with the fractions of day 7 whey from vaccinated cows which, by the Mancini test, contained only IgG (Figure 4.5). The IgM and IgA K99 antibodies if present must have been at such low concentrations that they were undetectable.

The manufacturers, Miles Laboratories, stated in their descriptive literature that the reagents were monospecific when tested by immunoelectrophoresis and immunodiffusion against bovine sera and whey. It is possible that the ELISA because of its sensitivity may detect cross reactions of the conjugates by anti-light chain reactions on immunoglobulin molecules which are not normally detected by immunoelectrophoresis or immunodiffusion. The antisera may have been prepared by the immunisation of rabbits with purified IgG, IgA or IgM. In such circumstances heavy chain specific and cross-reacting anti-light chain antibodies may have been produced. Thus to obtain specific ELISA reagents for the detection of IgG, IgA and IgM, heavy chain specific conjugates should be used. Such reagents may be prepared by monoclonal antibody production or absorption of light chain specific antibodies from a mixture to leave only heavy chain specific antibodies.

Because of their apparent non-specific reactions the anti-bovine IgA and IgM conjugates were not used any further in the sandwich ELISA. Only the anti-bovine IgG conjugate was used for experiments described in Chapter 4, Section B, but one has to take into consideration that this reagent may be reacting non-specifically with IgM and IgA K99 antibodies.

The immunoglobulin class specific conjugates were to be used for analysis of IgG, IgA and IgM anti-K99 antibody responses in colostrum and milk from vaccinated cows in Chapter 4, Section B. There are 2 reports of the application of the ELISA in this way using commercially produced antisera (Anderson, Rowe, Taylor and Crowther, 1982; Abu-Elzein and Crowther, 1981). The antisera used in these studies were stated to be specific for heavy chain determinants by the manufacturers, and were found during the work performed to be class specific. McLean, Sonza and Holmes (1980) found their anti-human IgG, IgA and IgM conjugates to cross react with immunoglobulins of other classes but overcame this by diluting out the non-specific factors. If such an approach was adopted for the anti-IgA and IgM conjugates used in this study, then their specific activity may be lost.

The blocking tests on fractions of day 1 and day 7 whey confirmed the presence of K99 antibody activity indicated by the rabbit anti-bovine immunoglobulin conjugates. A strong blocking effect apparent in the IgM rich region decreased in the IgA rich region but increased in the long IgG rich region for fractionated day 1 whey. The blocking effects observed for fractionated day 7 whey in the ELISA suggest that the K99 antibody levels (specifically IgG) were high in day 7 milk and hence may be responsible for the prolonged K99 antibody secretion pattern seen in the milk of vaccinated cows.

The blocking ELISA was useful for determining the K99 antibody titres of rabbit antisera. The vaccination schedule used to immunise rabbits (Chapter 2) proved to be successful in producing high K99 antibody titres in the range 1500 to 2800. The specific K99 antiserum obtained from Dr. W.H. Jansen, which was used as coating and conjugated

antibody for the ELISA, had a titre of 1800 thus confirming the potency of this antiserum.

As there is immunological cross reactivity between bovine and ovine immunoglobulins it was possible to use the sandwich ELISA to detect ovine K99 antibodies. A titre of 6400 was found for the gnotobiotic lamb serum using rabbit anti-bovine IgG conjugate. As this antiserum is specific for K99 it may be used as a reagent (i.e. coating antibody and conjugated antibody) in the ELISA for the detection of K99 antigen.

CHAPTER 4 SECTION B

ATTEMPTS TO DEVELOP A COMBINED VACCINE TO PROTECT NEONATAL CALVES AGAINST ETEC AND ROTAVIRUS INFECTIONS

INTRODUCTION

The aim of this study was to produce a dual component vaccine containing a crude K99 antigen extract and rotavirus antigen. The intention was to stimulate production of antibodies to these components in pregnant cows, and thereby enhance protection of their offspring through the secretion of long lasting, specific antibodies in the colostrum and milk.

The experiments were carried out in collaboration with Dr. L.K. Nagy (Wellcome Research Laboratories, Beckenham, England) who provided both the crude K99 antigen component of the vaccine and sera from laboratory animals treated with the vaccine. The rotavirus component of the vaccine was provided by Dr. D.R. Snodgrass. The results which relate to ETEC, the author's component in the experiments are presented in full, but those pertaining to rotavirus are merely summarised.

EXPERIMENT 1: THE K99 ANTIBODY RESPONSE OF LABORATORY ANIMALS AFTER TREATMENT WITH A K99/ROTAVIRUS VACCINE

MATERIALS AND METHODS

The animal experiments were performed by Dr. L.K. Nagy. The doses of vaccine used are shown in Table 4.6. Guinea pigs (Groups 1 to 7), rats (Groups 8 to 11) and weaned mice (Groups 15 and 16) were given a single intraperitoneal injection of oil adjuvant vaccine, and rats of Groups 12 to 14 a single injection of Alhydrogel

Table 4.6 K99 antibody titres measured by blocking ELISA of laboratory animals 5 weeks post injection with varying dose levels of a K99/rotavirus vaccine

| Animal Group ^a | Adjuvant | Units of K99/ dose (see Experiment 4) | Rotavirus dose TCID ₅₀ before inactivation (x10 ⁴) | Mean 50% K99 ELISA blocking titre as log ₁₀ (+se) |
|---------------------------|------------|---|---|--|
| Guinea pigs | oil | 30 | 6.00 | result undetermined |
| | " | 15 | 3.00 | " |
| | " | 6 | 1.20 | 2.75 ± 0.16 |
| | " | 3 | 0.60 | 2.43 ± 0.20 |
| | " | 1.2 | 0.24 | 2.32 ± 0.11 |
| | " | 0.6 | 0.12 | 1.08 ± 0.07 |
| | " | 0.24 | 0.05 | <1.0 |
| Rats | oil | 30 | 6.00 | result undetermined |
| | " | 6 | 1.20 | " |
| | " | 1.2 | 0.24 | 1.56 ± 0.59 |
| | " | 0.24 | 0.05 | 1.0 ± 0.27 |
| | Alhydrogel | 20 | None | <1.0 |
| Mice | " | 4 | None | <1.0 |
| | " | 0.8 | None | <1.0 |
| | oil | 30 | 6.00 | 2.51 |
| | " | 6 | 1.20 | 1.60 |

a; 5 animals/group. b; sera pooled from 10 mice.

adjuvant vaccine by the same route. The method of preparation of the vaccines is described in Experiment 4 of this section.

Sera obtained at 5 weeks post vaccination were examined for K99 antibodies by the blocking ELISA test (Chapter 4, Section A).

RESULTS

The mean 50% blocking ELISA titres are shown in Table 4.6. For technical reasons it was not possible by this test to establish the full serological response of guinea pigs and rats given high doses of K99 antigen in oil adjuvant but otherwise it was apparent that K99 antibody production was dose related in all 3 laboratory animal species tested. The presence of rotavirus antigen did not have any apparent effect on the response to K99 antigen.

There was no serological response of rats vaccinated with 0.8, 4 and 20 units of K99 antigen in Alhydrogel adjuvant.

EXPERIMENT 2: THE K99 ANTIBODY RESPONSE OF STEERS TO VARIOUS DOSES OF A K99/ROTAVIRUS VACCINE

INTRODUCTION

This experiment was performed to establish the optimal K99 vaccination dose and to identify the time of peak antibody response following vaccination.

MATERIALS AND METHODS

The following animal experiment was carried out by Dr. L.K. Nagy. Twenty-five steers, 1 to 3 years of age were divided into 3 groups. Group 1 steers received 15 units of K99 antigen and 3.77×10^6 50% tissue culture infective doses (TCID₅₀) of rotavirus before inactivation; Group 2 steers, 75 units of K99 and 1.51×10^5

TCID₅₀ rotavirus and Group 3 steers, 375 units of K99 and 7.54×10^5 TCID₅₀ rotavirus. All preparations were in oil adjuvant and were injected as a 1ml dose intramuscularly. Sera were obtained before vaccination and every month for 5 months post vaccination. The sera were assayed for K99 antibodies by the sandwich ELISA (Chapter 4, Section A).

RESULTS

Thirteen of the 25 steers had K99 antibody titres before vaccination (Appendix 1). Post vaccination titres of all 3 vaccine groups peaked at two months post vaccination but were high over the full 5 month period (Table 4.7). Groups 2 and 3 (75 and 375 units of K99 antigen respectively) gave very similar antibody responses, but Group 1, which received 15 units of K99 antigen had significantly lower (T-test) antibody titres than Groups 2 and 3 from 2 months post vaccination onwards (Table 4.7).

EXPERIMENT 3: THE K99 ANTIBODY RESPONSE OF PREGNANT COWS TO VARIOUS DOSES OF A K99/ROTAVIRUS VACCINE INJECTED INTRAMUSCULARLY OR SUBCUTANEOUSLY

MATERIALS AND METHODS

Thirty pregnant cows were randomly divided into 5 groups of 6 animals each. Three cows in each group were injected intramuscularly (IM) and 3 subcutaneously (SC) with one of the 5 vaccine preparations shown in Table 4.8. The range of K99 antigen content, 4 to 65 units, was selected on the basis of the findings obtained in Experiment 2, in which moderate antibody responses were observed with 15 units of K99 antigen. The proportions of antigen and oil adjuvant were the same in all vaccines, which were prepared as described in Experiment 4.

Table 4.7 K99 serum antibody response over a 5 month period of steers treated with one of 3 different dose levels of an oil adjuvant vaccine as measured by the sandwich ELISA

| Group | No of steers | Units of K99 antigen administered | Pre-bleed | Mean K99 antibody titre ($\log_{10} \pm \text{se}$) | | | | |
|-------|--------------|-----------------------------------|-----------------------|---|-------------------------|-------------------------|-------------------------|-------------------------|
| | | | | Months post vaccination | | | | |
| | | | | 1 | 2 | 3 | 4 | 5 |
| 1 | 10 | 15 | 2.15 \pm 0.11 | 3.50 \pm 0.03 | 3.50 \pm 0.03 | 3.40 \pm 0.04 | 3.30 \pm 0.06 | 3.23 \pm 0.06 |
| 2 | 10 | 75 | 1.89 \pm 0.11 | 3.55 \pm 0.04 | 3.60* \pm 0.30 | 3.55* \pm 0.03 | 3.52** \pm 0.05 | 3.48** \pm 0.05 |
| 3 | 5 | 375 | 2.16 \pm 0.11 | 3.62 \pm 0.04 | 3.67** \pm 0.04 | 3.61** \pm 0.05 | 3.62** \pm 0.03 | 3.57** \pm 0.02 |

* $P < 0.05$

** $P < 0.01$ with respect to Group 1.

Table 4.8 K99 antibody response of pregnant cows vaccinated intramuscularly (IM) or subcutaneously (SC) with one of 5 different dose levels of oil adjuvant vaccine as measured by the sandwich ELISA

| Group ^a | Units of K99 antigen | Rotavirus (TCID ₅₀) before inactivation | No of cows with negative Pre-vacc titres (<2.0) | Mean K99 antibody titre as log ₁₀ + se of animals vaccinated | |
|--------------------|----------------------|---|---|---|---------------------------------|
| | | | | IM | SC |
| 1 | 65 | 10 ^{6.2} | 4 | 3.09 ^{**} + 0.19 | 3.70 + 0.03 |
| 2 | 32.5 | 10 ^{5.2} | 5 | 3.57 ^{***} + 0.005 | 3.23 + 0.17 |
| 3 | 16.0 | 10 ^{4.2} | 2 | 3.03 [*] + 0.08 | 2.79 [*] + 0.20 |
| 4 | 8.0 | 10 ^{3.2} | 5 | 3.07 ^{**} + 0.22 | 2.27 ^{**} + 0.28 |
| 5 | 4.0 | 10 ^{2.2} | 4 | 2.15 + 0.20 | 2.67 ^{**} + 0.16 |

a; 6 cows per group.

* P<0.05) Bartlett's F test

** P<0.01) Analysis of variance with respect to Group 5 for IM

*** P<0.001) injections and with respect to Group 1 for SC injections.

Each cow was injected once with 1ml of vaccine. Cows were bled before and 4 weeks after vaccination; sera were tested by the sandwich ELISA.

RESULTS

The results are summarised in Table 4.8. Ten cows had K99 serum antibody titres before vaccination. All vaccine preparations gave a rise in K99 antibody titres irrespective of the route of administration. No significant differences in antibody titres were observed between SC and IM injected cows in any of the 5 groups except Group 4, in which titres of SC infected cows were significantly lower ($P < 0.05$) than those injected IM. The IM vaccinated cows of Group 5 produced a significantly lower response than was obtained from all other animals immunised by this route. Conversely, the SC immunised cows of Groups 1 and 2 produced a significantly higher response than all other SC injected animals. No consistent dose response was observed, although the antibody response tended to fall at lower doses of K99 antigen. The highest K99 antibody titres were achieved in cows vaccinated IM with vaccine 2 (mean titre $10^{3.57}$) and vaccinated SC with vaccine 1 (mean titre $10^{3.70}$).

EXPERIMENT 4: VACCINATION OF PREGNANT COWS WITH A K99/ROTAVIRUS

VACCINE AND CHALLENGE OF NEWBORN CALVES WITH ETEC

MATERIALS AND METHODS

Animals

Twenty-four cows of 7 to 15 years old were allocated to treatment groups 4 to 10 weeks prior to calving. They were not housed until 4 weeks after the first vaccine dose.

Calves were challenged with ETEC between 6 and 18 hours after birth. After challenge cow/calf pairs from different treatment groups were kept separated from other pairs for at least 3 days to prevent cross suckling, then moved to a communal pen separate from the uncalved cows.

Titration of K99 antigen

The method was derived from the in vitro assay used to quantitate K88 antigens of E. coli (Nagy, Walker, Bhagal and MacKenzie, 1978) and was carried out by Dr. L.K. Nagy. The test was based on the capacity of K99 antigen to adsorb specific antibodies from a standard antiserum; residual K99 antibody was then measured by PHA. The standard antiserum was produced in a pig vaccinated with a sterile culture supernate of ETEC strain 09: K35 (A):K99. The serum was adsorbed to remove detectable 09 and K35 (A) agglutinins. The highest dilution of test sample which prevented agglutination of the K99 'indicator' antigen by the standard antiserum gave an estimate of the test sample K99 antigen content in agglutinin units (units).

Vaccines

(a) Alhydrogel adjuvant vaccine

The K99 component was prepared from the culture supernate of ETEC strain B41 (0101:K-:K99) by Dr. L.K. Nagy. The E. coli was grown in a synthetic medium (information restricted by the Wellcome Foundation) which contained selected amino acids, trace salts and lactose, and was buffered with phosphates to pH 7.5. After 8 to 10 hours incubation at 37 °C in an aerated vessel the culture was inactivated in situ at 60 °C for 30 minutes. Cells were separated

aseptically from the supernate by centrifugation. After titration of the K99 antigen content of the sterile supernate by the PHA test, Alhydrogel (aluminium hydroxide, Superfos Export Company, Copenhagen, Denmark) was added to a final concentration of 20%.

Tissue culture adapted rotavirus was prepared and inactivated by Dr. D.R. Snodgrass as previously described (Snodgrass, Fahey, Wells, Campbell and Whitelaw, 1980). The rotavirus and K99 components were blended together aseptically with Alhydrogel in the proportions 1:1:8 so that each 1ml of the combined vaccine contained 15 units of K99 antigen and $10^{5.4}$ TCID₅₀ rotavirus before inactivation.

(b) Oil adjuvant vaccine

The K99 component was produced by Dr. L.K. Nagy from ETEC strain B41 (0101:K-:K99) grown on 5% horse blood agar at 37 °C for 18 hours. The growth was harvested in sterile saline and concentrated by centrifugation so that it contained 2.5×10^{11} cfu/ml. The concentrate was homogenised in 20ml volumes (Silverson) for 4 minutes at 4 °C. Cells were removed from the supernate by centrifugation at 20,000g at 4 °C for 30 minutes, 0.1% Merthiolate was added and the preparation heated at 60 °C for 30 minutes. The sterility of this crude K99 extract was confirmed and its K99 antigen content titrated by the PHA test before use.

Rotavirus and K99 antigen were mixed with Tween 80 (0.2% v/v final concentration) then emulsified in a 1:2 proportion with oil adjuvant (90% Marcol 52 (Esso), 10% Arlacel (Sandria Chemicals)). Each 1ml of vaccine contained 60 units of K99 and $10^{5.2}$ TCID₅₀ rotavirus before inactivation.

Vaccination of pregnant cows

Two groups of 6 cows each were vaccinated once 4 to 10 weeks before parturition with either 2ml or 0.5ml of oil adjuvant vaccine given by deep intramuscular injection into the neck. One group of 6 cows was similarly vaccinated at the same time and site on 2 occasions 28 days apart with 4ml of Alhydrogel adjuvant vaccine. Six unvaccinated cows constituted control animals although 4 of these had been vaccinated with a rotavirus/coronavirus vaccine in their previous pregnancy.

K99 serology

(a) Detection of serum and whey K99 antibodies by sandwich ELISA

The method was performed as described previously (Chapter 4, Section A).

(b) The passive haemagglutination assay (PHA)

The assay was performed by Dr. L.K. Nagy. Pyruvic acid stabilised sheep erythrocytes (Hirata and Brandriss, 1968) were sensitised with K99 antigen extracted from ETEC strain 08:K85ab:K99. A suspension of erythrocytes in 0.1M acetate buffer pH 4.5 was coated to saturation with K99 derived by the method of Morris, Stevens and Sojka (1977), washed 5 times in PBS pH 7.5 and resuspended to 1% v/v. Test sera and whey samples were absorbed with an equal volume of packed unsensitised erythrocytes for 18 hours at 4 °C to remove non-specific haemagglutinins. Serial doubling dilutions of serum or whey in 0.3% formol saline were prepared in microtitre plates followed by the addition of an equal volume (0.025ml) of sensitised erythrocytes. The agglutination pattern was read after 18 hours incubation at 37 °C. Samples were tested on only one occasion.

Rotavirus serology

Sera and whey from colostrum and milk samples were assayed for neutralising antibody to tissue culture adapted calf rotavirus on bovine embryo kidney cells or MA104 cells grown in microtitre plates by Miss I. Campbell (Moredun Research Institute).

Toxicity of K99 components in the K99/rotavirus vaccine

The K99 vaccine preparations were screened for toxicity by injecting groups of 10 mice i.p. with 15 units of Alhydrogel absorbed K99, or with 180 units of crude K99 antigen extract.

ETEC challenge inoculum

E. coli strain B44 (09:K30:K99) was grown in TSB for 8 hours, then subcultured on minca-Isovitalex agar for 18 hours at 37 °C. The bacterial growth was suspended in PBS with dimethyl sulphoxide added to 10% (v/v) final concentration and stored in 10ml aliquots at -70 °C. The viable count before freezing was 4.5×10^9 cfu/ml.

The calves were not challenged experimentally with rotavirus.

Assessment of the pathogenicity of the ETEC challenge strain

(a) Animals

Five Friesian calves obtained from a farm as soon after birth as possible were housed together in an isolation room and challenged at 6 to 18 hours old. Calves were fed twice daily with 1 litre of reconstituted evaporated milk.

(b) ETEC challenge

Two calves were inoculated orally with 2×10^{10} cfu, 1 calf with 4×10^{10} cfu and 2 calves with 8×10^{10} cfu of ETEC strain B44. One of the calves inoculated with 2×10^{10} cfu was killed in extremis at 48 hours of age and intestinal material was taken as described in Chapter 2.

Vaccine trial challenge

Each calf was inoculated orally with 10ml of the challenge inoculum thawed immediately before use. The mean inoculum titre was 4.1×10^{10} cfu/10ml (range 3.0 to 6.8×10^{10} cfu/10ml). No significant decrease in the titre of aliquots was observed over the 2 month experimental period. The enterotoxigenicity of the stored organisms was confirmed periodically by the infant mouse test for STa (Chapter 2). The possession of the K99 antigen was confirmed by slide agglutination (Chapter 2).

Microbiological examination of faeces

Faeces samples were examined for rotavirus by ELISA and for Cryptosporidium sp by Giemsa staining (Chapter 2). Samples were cultured on MacConkey agar and minca-Isovitalex agar as described in Chapter 2. Five colonies from the growth on minca-Isovitalex agar were tested by the slide test for K99 antigen and 3 colonies from the growth on MacConkey agar were examined by the slide test using diluted rabbit antiserum to strain B44.

At intervals faecal swabs were tested for Salmonella sp by overnight enrichment in SSB and for Campylobacter sp by growth on 5% sheep blood agar with Skirrows antibiotic supplement (Oxoid) in microaerophilic conditions.

Clinical observations

Calves were weighed at 1, 2 and 3 days old. All calves were examined clinically at least once a day for 6 days and assigned a clinical score on a subjective scale similar to that used by Myers (1980) : (1) normal, faeces firm; (2) transient diarrhoea within 24

hours of inoculation lasting only a few hours; (3) severe watery diarrhoea, calf becoming dehydrated and dull; (4) severe watery diarrhoea, calf too weak to stand with death ensuing.

Sampling

Serum samples were collected from each cow before vaccination, 28 days post vaccination, at parturition and 28 days, between 56 to 105 days and between 260 to 310 days after parturition. Colostrum and milk samples were collected on days 1, 3, 7, 14, 28 and between 56 to 105 days after calving and whey prepared from these using rennet. Calves were bled at 3, 28, between 56 to 105 and between 160 to 210 days of age. Faecal samples collected daily from calves for 6 days after birth were examined microbiologically, and the remainder was dried at 100 °C to constant weight for dry matter estimation.

RESULTS

K99 antibodies in the sera of vaccinated and unvaccinated control cows determined by the sandwich ELISA

Six cows had low pre-existing serum antibody levels (mean titre 2.17) while the remaining 18 cows were negative (titre <2.0). Mean titres to the K99 antigen at 28 days post vaccination were significantly increased by both oil adjuvant vaccines, but not by the single injection of Alhydrogel adjuvant vaccine (Table 4.9). All vaccine regimes had significantly increased antibody titres ($P < 0.001$) at calving, the mean titres at calving induced by both oil adjuvant vaccines given in Table 4.9 did not differ significantly from each other, but both were significantly higher ($P < 0.001$) than the titres of cows vaccinated with the Alhydrogel adjuvant vaccine. High K99 antibody levels were present in all sera of vaccinated cows at calving and 28 days after calving, with

Table 4.9 K99 antibody response in the sera of vaccinated and unvaccinated control cows as determined by the sandwich ELISA

| Vaccine ^a group | Mean K99 antibody titres as $\log_{10} \pm se$ | | | | | |
|-------------------------------|--|--------------------------------------|--------------------------------------|---------------------------------------|---------------------------------------|------------------------------------|
| | Pre- vaccination | 28 days post vaccination | At calving | Days post calving | | |
| | | | | 28 | 56-105 | 260-310 |
| Control | 1.78 \pm 0.09 | 1.77 \pm 0.08 | 1.77 \pm 0.08 | 2.68 ^b \pm 0.52 | 2.05 ^c \pm 0.24 | 2.02 ^c \pm 0.15 |
| 2ml oil adjuvant | 1.82 \pm 0.13 | 4.16 ^{***} \pm 0.10 | 4.28 ^{***} \pm 0.07 | 4.65 ^{d***} \pm 0.06 | 3.50 ^{***} \pm 0.05 | 3.00 ^c \pm 0.37 |
| 0.5ml oil adjuvant | 1.96 \pm 0.12 | 3.92 ^{***} \pm 0.13 | 3.95 ^{***} \pm 0.14 | 3.74 \pm 0.10 | 2.52 ^{c***} \pm 0.32 | NT |
| Alhydrogel adjuvant | 1.70 | 2.11 \pm 0.22 | 2.93 ^{***} \pm 0.21 | 3.50 \pm 0.19 | 1.70 [*] | NT |

a; 6 cows per group.

b; 4 cows tested.

c; 5 cows tested.

d; 3 cows tested.

NT; not tested

* $P < 0.05$) Bartlett's F test

** $P < 0.01$) Analysis of variance, with respect to control values.

*** $P < 0.001$)

a subsequent decline thereafter which was most marked in the cows injected with Alhydrogel vaccine.

Control cows had seroconverted at 28 days post calving presumably to the challenge ETEC strain in the environment. It is also likely that the vaccinated cows had their K99 antibody titres boosted by exposure to the challenge strain.

K99 antibodies in colostrum and milk whey as measured by the sandwich ELISA

The results are shown in Table 4.10. Three control cows had low K99 antibody levels in colostrum but not in milk whey.

K99 antibody titres in colostrum and milk whey of cows treated with both oil adjuvant vaccines were significantly higher than those of unvaccinated controls, although titres declined slowly over the 84 days of observation. Antibody titres in Alhydrogel vaccinated cows were also increased in colostrum and in milk whey at 3 days post calving but were undetectable thereafter. Figure 4.9 shows the K99 antibody titres in whey from the 4 groups of cows studied.

K99 antibodies in the sera of calves as measured by the sandwich ELISA

The serum K99 antibody titres in 3-day-old calves reflected the colostral antibody titres of their dams (Table 4.11). Calves sucking the cows in the 3 vaccine groups showed a decrease in K99 antibody titre over the 160 to 210 days studied. By this time all titres of calves in the Alhydrogel group were negative and only 2 of 6 calves in the 0.5ml oil group had K99 antibodies at low titres at this time. A single calf born to an unvaccinated control cow had serum K99 antibodies at 3 days old (titre of 2.24). At 28 days of age, 5 of the 6

Table 4.10 K99 antibody levels in the whey of vaccinated and unvaccinated cows as determined by the sandwich ELISA

| Vaccine ^a group | Mean K99 antibody titres ($\log_{10} \pm \text{se}$) on days post calving | | | | | |
|-------------------------------|---|--------------------------------------|--------------------------------------|--------------------------------------|--------------------------------------|--------------------------------------|
| | 1 | 3 | 7 | 14 | 28 | 56-105 |
| Control | 2.04 \pm 0.17 | 1.70 | 1.70 | 1.70 | 1.70 | 1.70 |
| 2ml oil adjuvant | 4.51 ^{***} \pm 0.07 | 4.10 ^{***} \pm 0.11 | 3.56 ^{***} \pm 0.21 | 3.48 ^{***} \pm 0.23 | 3.07 ^{***} \pm 0.13 | 2.91 ^{***} \pm 0.15 |
| 0.5ml oil adjuvant | 4.30 ^{***} \pm 0.09 | 3.88 ^{***} \pm 0.10 | 3.31 ^{***} \pm 0.14 | 2.83 ^{***} \pm 0.07 | 2.32 [*] \pm 0.33 | 2.56 ^{***} \pm 0.19 |
| Alhydrogel adjuvant | 3.06 ^{**} \pm 0.28 | 2.58 ^{**} \pm 0.27 | 1.84 \pm 0.12 | 1.70 | 1.70 | 1.70 |

a; 6 cows per group.

* $P < 0.05$)

** $P < 0.01$) Bartlett's F test

*** $P < 0.001$) Analysis of variance with respect to control values.

Comparison of 2ml oil group with 0.5ml oil group; only significant difference ($P < 0.01$) for 14 and 28 day whey.

Comparison of 2ml oil group with Alhydrogel group; significant differences ($P < 0.001$) for all samplings.

Comparison of 0.5ml oil group with Alhydrogel group; significant differences ($P < 0.001$) for all samplings except at 28 days ($P < 0.05$).

Table 4.11 K99 antibodies in the sera of calves sucking
vaccinated or unvaccinated control cows as
determined by the sandwich ELISA

| Vaccine group ^a | Mean K99 antibody titres ($\log_{10} \pm$ se) at days | | | |
|----------------------------|--|-------------------------------------|-----------------------|--------------------------------------|
| | 3 | 28 | 56-105 | 160-210 |
| Control | 1.81 \pm 0.12 | 3.20 \pm 0.33 | 3.74 \pm 0.03 | 2.83 \pm 0.40 |
| 2ml oil adjuvant | 4.28 ^{***} \pm 0.09 | 4.00 ^{**} \pm 0.18 | 3.48 \pm 0.09 | 2.30 \pm 0.22 |
| 0.5ml adjuvant | 4.17 ^{***} \pm 0.05 | 3.58 \pm 0.26 | 3.32 \pm 0.13 | 1.82 ^{***} \pm 0.08 |
| Alhydrogel adjuvant | 2.29 [*] \pm 0.30 | 2.65 \pm 0.17 | 2.38 \pm 0.38 | 1.70 ^{***} |

a; 6 calves per group

* $P < 0.05$) Bartlett's F test

** $P < 0.01$) Analysis of variance with respect to control

*** $P < 0.001$) values.

Comparison of 2ml oil group with 0.5ml oil group;
 significant difference observed ($P < 0.05$) at 160-210 days.

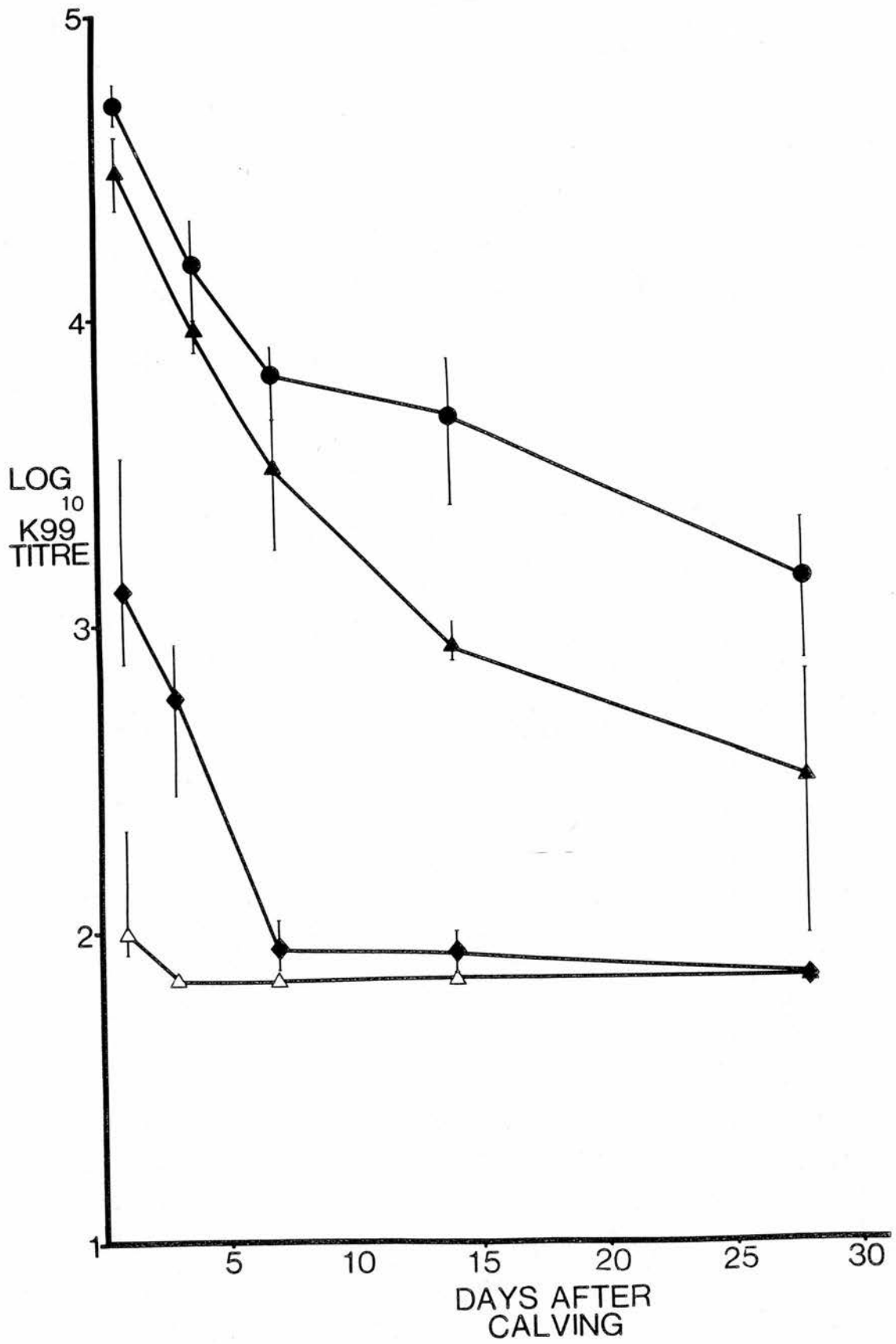
Comparison of 2ml oil group with Alhydrogel group;
 significant differences observed ($P < 0.01$) at all ages.

Comparison of 0.5ml oil group with Alhydrogel group;
 significant differences observed, $P < 0.001$, $P < 0.05$ and
 $P < 0.01$ for days 3, 28 and 56-105 respectively.

Figure 4.9 K99 antibody levels in whey from vaccinated and unvaccinated cows as determined by the ELISA.

- 2ml oil adjuvant vaccinated cows.
- ▲—▲ 0.5ml oil adjuvant vaccinated cows.
- ◆—◆ Alhydrogel adjuvant vaccinated cows.
- △—△ unvaccinated cows.

Standard errors are also given.



control calves had seroconverted to K99 antigen, their levels reaching a maximum at 56-105 days. The antibody levels of control calves at 160 to 210 days were significantly higher than those of calves from vaccinated dams.

Microbiological examination of faeces

Rotavirus was detected in the faeces of one 2-day-old control calf with diarrhoea, and in 3 clinically normal 2-day-old calves born to vaccinated cows. Cryptosporidium sp, Salmonella sp and Campylobacter sp were not detected in any faeces tested.

Isolated coliforms were tested for K99 antigen and for agglutination in antiserum to the challenge strain B44 for 6 days post challenge (Table 4.12, Appendix 2). E. coli strains positive by slide agglutination in adsorbed K12:K99 serum were detected significantly less frequently in all vaccinate groups than in the control group (Table 4.12 and Figure 4.10). However, only the 0.5ml oil and Alhydrogel groups differed significantly from the control group in the excretion rates of isolates which agglutinated with B44 antiserum. The number of B44 positive isolates was significantly higher than the number of K99⁺ isolated in all 3 vaccine groups ($P < 0.05$) but not in the control group. There was also intra-group variation in the levels of excretion of organisms (Appendix 2).

K99 antibody response in the sera and whey of vaccinated and unvaccinated cows by PHA

Assay of sera and whey by PHA confirmed the results found by sandwich ELISA. The results are shown in Table 4.13.

Rotavirus immunological response

Results from the 4 unvaccinated control cows which had been vaccinated in a previous pregnancy are excluded from these results; to

Table 4.12 Faecal excretion over 6 days of ETEC strain B44 after challenge of calves

| Vaccine group ^a | Mean percentage (\pm se) of <u>E. coli</u> isolates obtained over 6 days agglutinated by antiserum to | |
|----------------------------|--|--------------------------|
| | K99 | B44 |
| Control | 83.33 \pm 7.33 | 81.00 \pm 6.03 |
| 2ml oil adjuvant | 49.00* \pm 13.09 | 63.50 \pm 13.75 |
| 0.5ml oil adjuvant | 16.33*** \pm 6.06 | 38.50** \pm 7.86 |
| Alhydrogel adjuvant | 30.67** \pm 7.85 | 45.30* \pm 6.79 |

a; 6 calves per group.

* P<0.05) Students 'T' test

** P<0.01) Analysis of variance with respect to control values.

*** P<0.001)

Table 4.13 Serum and whey K99 antibody titres from vaccinated and unvaccinated control cows as determined by the PHA test

| Vaccine group ^a | Mean K99 antibody titres (\log_{10}) in | | | | | | | |
|----------------------------|---|----------------------|---------------------------|--------------------|--------------------|--------------------|-------------------|--|
| | Sera | | whey (days after calving) | | | | | |
| | At calving | 28 days post calving | 1 | 3 | 7 | 14 | 28 | |
| Control | 1.83 | 2.00 | 2.23 | 1.87 | 1.70 | 1.75 | 1.70 | |
| 2ml oil adjuvant | 4.47 ^{*+} | 4.61 ^{*+} | 4.96 ^{*+} | 3.88 ^{*+} | 3.75 ^{*+} | 3.15 ^{*+} | 2.67 [*] | |
| 0.5ml oil adjuvant | 3.73 ^{*+} | 3.91 [*] | 5.16 ^{*+} | 3.68 ^{*+} | 3.28 ^{*+} | 2.66 ^{*+} | 2.60 [*] | |
| Alhydrogel adjuvant | 2.44 ^{*+} | 2.90 [*] | 3.33 [*] | 2.47 [*] | 1.90 | 2.05 | 1.78 | |

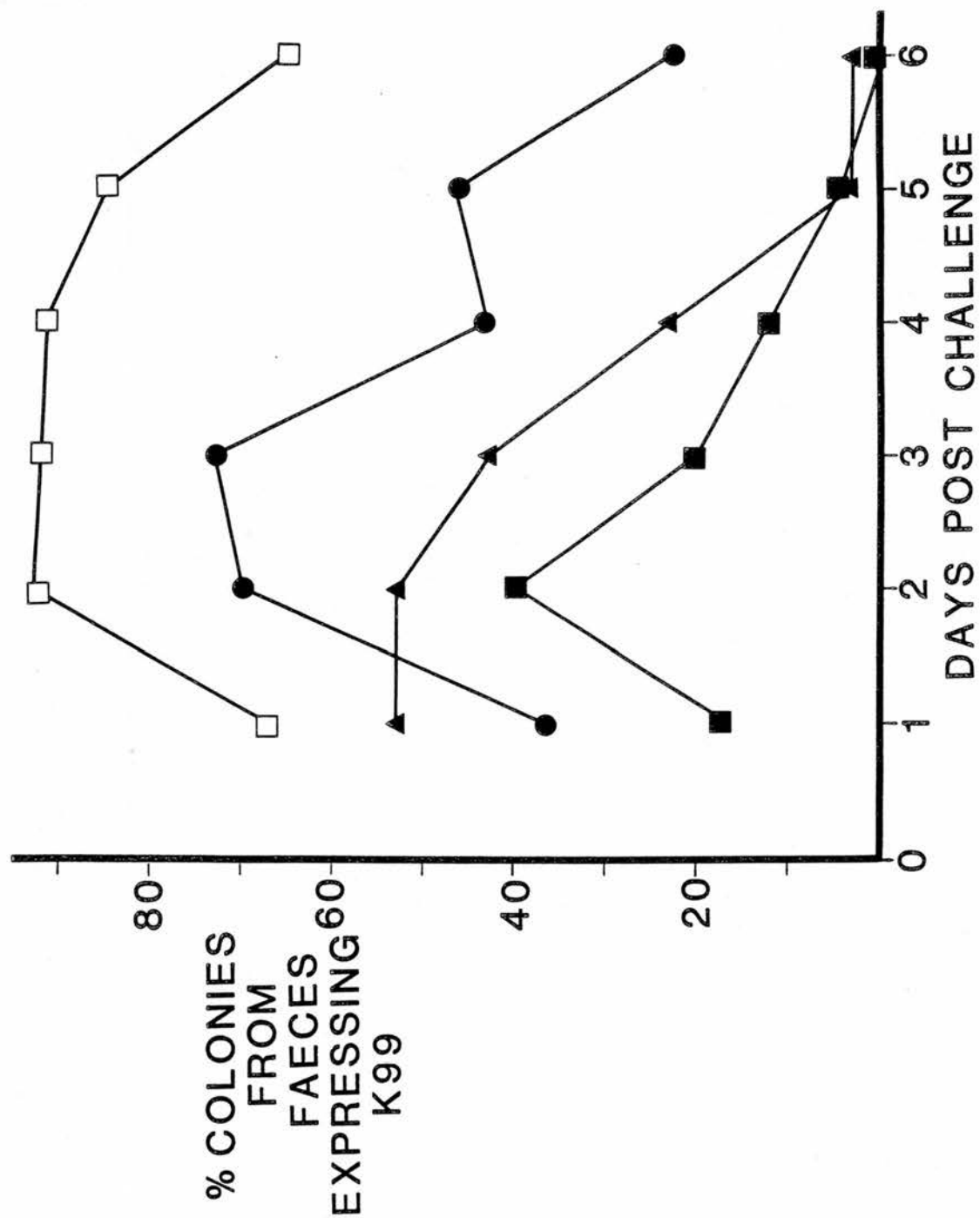
a; 6 cows per group.

* Significant differences between vaccinated and controls.

+ Significant differences between Alhydrogel and oil adjuvant groups.

Figure 4.10 The mean faecal shedding of K99⁺ E. coli after challenge of calves at 6 to 18 hours of age with ETEC strain B44.

- — □ calves from unvaccinated cows.
- — ● calves from 2ml oil adjuvant vaccinated cows.
- ▲ — ▲ calves from Alhydrogel adjuvant vaccinated cows.
- — ■ calves from 0.5ml oil adjuvant vaccinated cows.



compensate, data from 7 unvaccinated cows from the same farm have been included in the controls. All cows had pre-vaccination serum antibodies to rotavirus. Serum, colostrum and milk antibody titres of cows receiving oil adjuvant vaccine and colostral antibodies of Alhydrogel vaccinated cows were significantly raised (Table 4.14).

Assessment of the pathogenicity of the challenge ETEC strain

All calves developed severe watery diarrhoea 12 to 24 hours post challenge. Two calves which received 8×10^{10} or 2×10^{10} cfu died and one which received 2×10^{10} cfu was killed in extremis. These calves were extremely dehydrated and had collapsed before death. The remaining calves became dehydrated but survived after oral fluid replacement therapy.

Bacterial counts in the small intestine of the calf killed in extremis ranged from 1 to 2.5×10^{10} cfu/0.1g mucosal scrapings in sites taken from the jejunum to terminal ileum, and indirect immunofluorescence studies showed extensive bacterial colonisation of the intestine from mid gut to ileum (Figure 4.11). Light, transmission and scanning electron microscopy confirmed the extent of colonisation of the ileum (Figures 4.12 to 4.14).

Response of vaccine trial calves to challenge with ETEC

After challenge with ETEC strain B44, 5 of 6 control calves developed acute enteric disease (disease rating 3 or 4) characterised by profuse watery diarrhoea, dullness and dehydration (Table 4.15). The severity of the disease in the control calves was indicated by the mean body weight loss of 5.7% and a mean minimum faecal dry matter of 8.45%. One calf died 48 hours post challenge.

Table 4.14 Neutralisation antibody titres to rotavirus in the sera and whey of vaccinated and unvaccinated control cows

| Mean neutralisation titres as log ₁₀ in | | | | | | | | | |
|--|-----------------|--------------------------|------------|---------------------------|-------|--------|------|--------|--|
| Vaccine group ^a | Sera | | | Whey (days after calving) | | | | | |
| | Pre-vaccination | 28 days post vaccination | At calving | 1 | 3 | 7 | 14 | 28 | |
| Control | 2.51 | 2.66 | 2.36 | 2.81 | 2.05 | 2.02 | 1.83 | 1.70 | |
| 2ml oil adjuvant | 2.66 | 3.19 | 3.46* | 4.06** | 3.51* | 2.56** | 2.51 | 2.26** | |
| 0.5ml oil adjuvant | 2.81 | 3.71 | 3.65* | 3.96** | 3.46* | 2.91** | 2.41 | 2.43** | |
| Alhydrogel adjuvant | 2.66 | 3.33 | 3.29 | 3.51* | 2.81 | 2.36 | 2.15 | 2.02 | |

a; 6 cows per group, except for control group, 9 cows.

* P<0.05) Student 't' test

** P<0.01) Analysis of variance with respect to control values.

Table 4.15 Clinical assessment of calves challenged with EIEC

| Vaccine group ^a | Assessment at 48 hr. post challenge: number of calves with disease rating | | | | mean weight at 48 hr. | mean minimum faecal dry matter content ^b (%) |
|----------------------------|---|---|---|---|--------------------------|---|
| | 1 | 2 | 3 | 4 | mean weight at birth (%) | |
| Control | | 1 | 4 | 1 | 94.30 + 3.18 | 8.45 + 1.87 |
| 2ml oil adjuvant | 3 | 3 | | | 107.50** + 1.39 | 28.68** + 2.90 |
| 0.5ml oil adjuvant | 3 | 3 | | | 106.90** + 1.45 | 24.94** + 2.56 |
| Alhydrogel adjuvant | 3 | 3 | | | 103.20* + 1.25 | 21.82** + 1.79 |

a; 6 calves per group.

b; Minimum value recorded for each calf during the 5 days following EIEC challenge.

* P<0.05) Students 't' test

** P<0.01) Analysis of variance with respect to control values.

Figure 4.11 Cryostat section of calf ileum showing extensive surface fluorescence. Staining was by antiserum raised against E. coli strain B44 and an anti-species immunoglobulin conjugated with fluorescein isothiocyanate. (FITC) (x370)

Figure 4.12 Adjacent villi in calf ileum, showing adherence of bacteria (arrowed) to mucosal surfaces.(x1480, oil immersion, haematoxylin and eosin (HE)).

94c

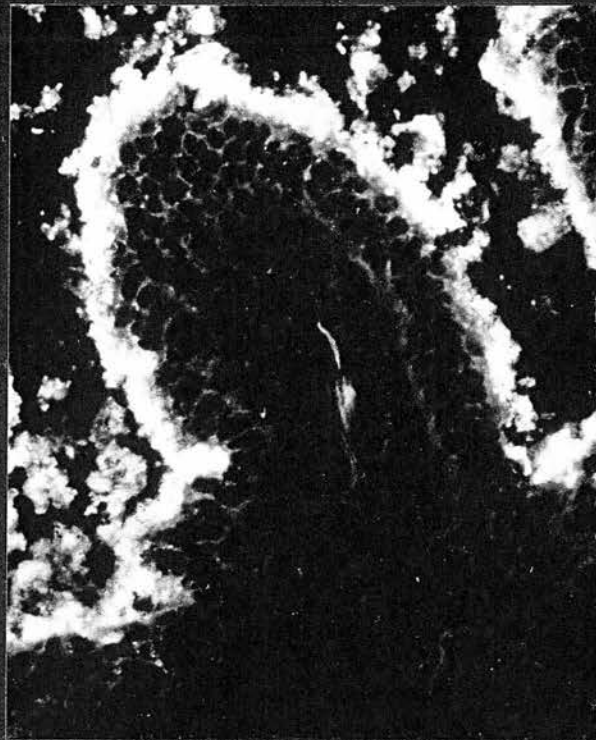


Figure 4.13a Scanning electron micrograph (SEM) of a villus
in calf ileum showing patchy bacterial adherence
on the mucosal surface.(x540)

Figure 4.13b SEM of the microvillous surface in calf ileum
showing the close association of bacteria with
micro-villi. (x20,000)

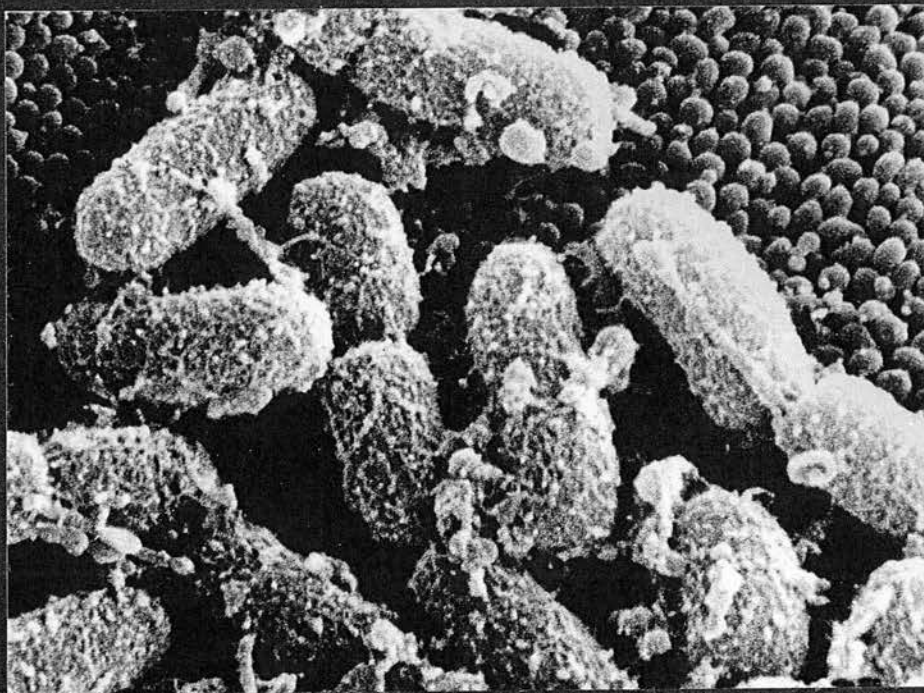
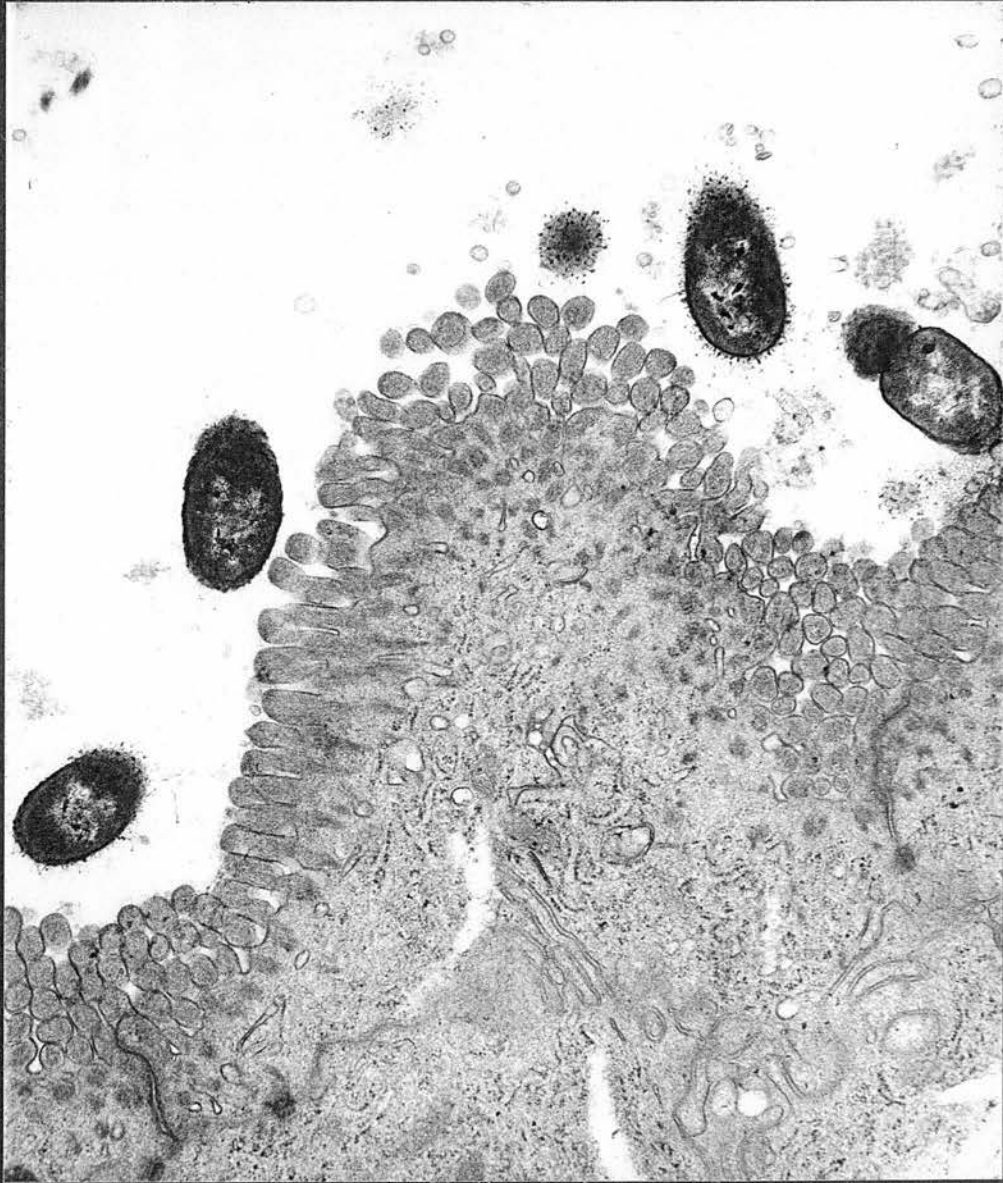


Figure 4.14 Transmission electron micrograph of the microvillous surface in calf ileum. Bacteria are apparently separated from the microvilli by small gaps. (x19,200)



In contrast, calves sucking vaccinated dams were only mildly affected and showed clinical signs of mild transient diarrhoea in 50% of the calves (disease rating 2). All calves gained weight, and the faecal dry matter did not fall below 16.3% in any individual.

Vaccine toxicity

The K99 antigen extract used in the vaccine did not produce any detectable toxic effects in mice, and all intramuscularly vaccinated cows remained normal.

EXPERIMENT 5: CALF BRUSH BORDER ANTI-ADHESION TEST USING POOLED COLOSTRAL WHEY FROM VACCINATED AND UNVACCINATED COWS

MATERIALS AND METHODS

Whey samples

Four pools of whey were prepared from equal volumes of day 1 whey samples obtained from the 6 cows of each group described in Experiment 4.

Brush border preparation

Brush borders were prepared from the intestine of a 9-day-old Ayrshire calf using the method of Sellwood, Gibbons, Jones and Rutter, (1975).

A length of distal small intestine which excluded ileum with Peyer's patches was excised from a freshly killed calf and washed free of its contents with cold saline. The intestine was ligated and filled with a solution containing 0.096M NaCl, 0.008M KH_2PO_4 , 0.0056M Na_2HPO_4 , 0.0015M KCl and 0.01M EDTA, pH 6.8 until slightly distended. The intestine was prevented from drying out externally by immersion in a similar solution in which 0.3M sucrose replaced EDTA. After incubation at room temperature for 20 minutes the

intestinal contents were discarded and the lumen half filled with sucrose buffer. Epithelial cells were detached into the lumen by gentle rubbing of the intestine between fingers. The intestinal contents were then collected.

All subsequent steps were performed at 4 °C. The epithelial cell suspension was centrifuged at 1200g for 10 minutes, the supernate discarded and the pelleted cells resuspended in 10 volumes of 0.005M EDTA pH 7.4 and then homogenised with a teflon tipped tissue grinder (clearance 0.015cm to 0.023cm) by moving the pestle up and down 6 times whilst it rotated at 900rpm. The homogenate was centrifuged at 300g for 5 minutes then suspended in 0.005M EDTA solution and the cycle repeated until the supernate became cleared of cellular fragments. The deposited brush borders were washed twice in Krebs-Henseleit buffer (K-H) pH 7.4 (0.12M NaCl, 0.14M KCl, 0.025M NaHCO₃ and 0.001M KH₂PO₄) and finally suspended in K-H buffer to provide approximately 10⁶ brush borders/ml. For storage an equal volume of brush borders in K-H buffer was added to glycerol and stored at -20 °C.

The anti-adhesion test

Serial 10^{0.5} dilutions of pooled whey samples in K-H buffer were incubated in 0.1ml volumes for 1 hour at 37 °C with 0.1ml K12:K99 E. coli grown overnight on 5% sheep blood agar and resuspended in K-H buffer to contain 10⁹ cfu/ml. Brush border preparation was added to pre-incubated bacteria in 0.1ml volumes (approximately 10⁵ brush borders), incubated at 37 °C for 30 minutes then scanned by phase microscopy. For each test 20 brush borders were examined for adhesion of bacteria. The diluted whey samples were also tested for their ability to agglutinate the K12:K99 strain in the slide test.

RESULTS

Serially diluted pooled whey from unvaccinated cows neither agglutinated E. coli strain K12:K99 in the slide test nor inhibited bacterial adhesion to calf brush borders (Table 4.16).

Whey from colostrum in all 3 vaccinate groups agglutinated the K12:K99 strain in the slide test at maximum dilutions of 1/1000, 1/300 and 1/10 for 2ml oil, 0.5ml oil and Alhydrogel vaccinated groups respectively. Total inhibition of adhesion of the K12:K99 strain to calf brush borders was seen at maximum dilutions of 1/10,000, 1/10,000 and 1/10 for 2ml oil, 0.5ml oil and Alhydrogel vaccinated groups respectively (Table 4.16).

DISCUSSION

The bivalent K99/rotavirus vaccine was effective in protecting calves against experimental diarrhoea produced by an enterotoxigenic E. coli strain B44.

The vaccine was originally made in two types of adjuvant, oil and aluminium hydroxide. Preliminary tests in rats (Experiment 1, Table 4.6) indicated that the Alhydrogel vaccine induced a weak K99 antibody response as measured by the blocking ELISA test even at a dose level of 20 units of K99 antigen. The injection of large quantities of K99 antigen in oil adjuvant into guinea pigs (30 and 15 units) and rats (30 and 6 units) resulted in sera which appeared to behave inconsistently in repeat tests. The reason for this inconsistency was not determined and was not a feature of other serum samples. This is a draw-back to the test, but despite this problem laboratory animals would appear to be potentially useful in potency testing of E. coli vaccines.

Table 4.16 Calf brush border anti-adhesion test and slide agglutination tests on pooled colostral whey from vaccinated and unvaccinated control cows

| Vaccine group ^a | Observations at whey dilution (\log_{10}) | | | | | | | | | |
|----------------------------|---|----------|----------|----------|----------|----------|----------|-----|-----|--|
| | 0.5 | 1.0 | 1.5 | 2.0 | 2.5 | 3.0 | 3.5 | 4.0 | 4.5 | |
| Control | ^b + (-) ^c | + | + | + | NT | NT | NT | NT | NT | |
| 2ml oil adjuvant | - (+) | - (+) | - (+) | - (+) | - (+) | - (+) | - (-) | + | + | |
| 0.5ml oil adjuvant | - (+) | - (+) | - (+) | - (+) | - (+) | - (-) | - (-) | + | + | |
| Alhydrogel adjuvant | - (+) | - (+) | + | + | + | NT | NT | NT | NT | |

a; Day 1 whey pooled from 6 cows in each group.

b; +, positive adhesion >5 organisms/brush border.

+ , reduced adhesion with 1 to 5 organisms/brush border.

-, negative adhesion <1 organism/brush border.

c; figures in parenthesis indicate slide agglutination test results with diluted whey; +, positive agglutination
-, negative agglutination

The oil adjuvant vaccine was injected IM into steers at three different doses. The results suggested that the optimal dose was of the order of 75 units, since the response to this was only slightly less than to a dose of 375 units, but markedly more than the response to 15 units. The peak K99 antibody response for each vaccine dose occurred at 2 months post vaccination; ideally, therefore pregnant cows should be vaccinated 6 to 10 weeks before parturition to ensure maximum K99 antibody response at calving.

The effect of vaccinating pregnant cows IM or SC with 5 different vaccine preparations was compared in Experiment 3. No significant differences were observed between IM or SC injections of any vaccine group. This observation was also made by Bagley and Call (1979) for a B44 bacterin. Also in Experiment 3 a decrease in K99 units tended to give a decrease in K99 antibody response. Recent work by Acres, Forman and Kapitany (1982) demonstrated that bacterin concentration was directly related to the levels of K99 antibody induced in cattle. This observation had previously been made in mice and rabbits in which the K99 antibody response was determined by the total K99 antigen injected (Forman, Acres and Kapitany, submitted for publication). Indeed this phenomenon has been shown in Experiment 1 of this section.

In the preliminary assessment of the B44 challenge strain, typical enterotoxigenic colibacillosis was reproduced in five Friesian calves less than 24 hours of age with doses of ETEC ranging from 2 to 8×10^{10} cfu. Severe disease and colonisation of the small intestine was observed as in previous studies (Bellamy and Acres, 1979; Pearson, McNulty and Logan, 1978). The ETEC challenge

strain B44 was originally isolated by Dr. H.W. Smith from an outbreak of neonatal calf diarrhoea and has been used by other workers as a challenge organism in vaccination experiments (Newman, Myers, Firehammer and Catlin, 1973; Wilson and Jutila, 1976; Acres, Isaacson, Babuik and Kapitany, 1979; Morris, Wray and Sojka, 1980). However, the challenge dose in the present trial (Experiment 4) was approximately 10-fold lower than those used previously. It was essential to challenge all calves before they were 24 hours old (as calves become progressively resistant to ETEC infections after this age) (Smith and Halls, 1967a) but after they had sucked colostrum, so that K99 antibodies were present in the gut of calves from vaccinated dams at the time of challenge. Thus the K99 antibodies may prevent colonisation of the intestine by inhibiting adhesion as shown by the vaccinate whey samples used in the in vitro test using brush borders (Experiment 5). This effect has been demonstrated for the anti-K99 activity of whey from ewes vaccinated with K99 antigen (Morris, Wray and Sojka, 1980). Hence the protective effect of colostrum is most probably due to the adhesion-inhibition properties of K99 antibodies.

All 3 vaccine regimes in Experiment 4 produced significant increases in K99 antibody titres in the sera of vaccinated cows. Pre-existing K99 antibody titres were apparent in only a few cows which is in contrast to the widespread occurrence of serum rotavirus antibodies in cows (Snodgrass, Fahey, Wells, Campbell and Whitelaw, 1980).

Three cows in the unvaccinated control group seroconverted to the K99 antigen in the post calving observation period, and detectable antibody persisted in the sera of some animals of this group for at

least 9 months. This finding supports a previous suggestion (Ellens, de Leeuw and Rozemond, 1978) that the ELISA has considerable potential for field investigations into the prevalence of K99⁺ ETEC infections.

K99 antibody titres in colostrum and milk whey of oil adjuvant vaccinated cows were significantly higher than those in the Alhydrogel adjuvant vaccinated and control groups. The Alhydrogel adjuvant vaccinated cows showed a significant increase in K99 antibodies in colostrum and day 3 milk but these antibodies rapidly declined to undetectable levels by 14 days. The increased colostral K99 antibody titres in the 3 vaccinated groups correlated well with the prevention of enterotoxigenic colibacillosis, and an antibody titre as low as $10^{2.76}$ was protective. Experimental evidence suggests that initial rotavirus infection may facilitate ETEC colonisation in calves 1 to 2 weeks of age (Runnels, Moon, Whipp, Matthews and Woode, 1980; Snodgrass, Smith and Krautil, 1982; Tzipori, Makin, Smith and Krautil, 1981) and such combined infections have been reported in the field (Moon, McClurkin, Isaacson, Pohlenz, Skartvedt, Gillette and Baetz, 1978; Acres, Laing, Saunders and Radostits, 1975). Thus it is clearly desirable to stimulate the production of high K99 antibody levels in milk for at least 14 days post-calving. This objective was achieved in this vaccination trial. In fact, K99 antibody titres in day 7 and 14 whey samples from both oil vaccinate groups were higher than the colostral K99 titres in the Alhydrogel vaccinated group.

The serum antibody titres of calves at 3 days old reflected the antibody levels found in their dam's colostrum. At 28 days old, titres in the two groups of calves from oil vaccinated cows started to decrease whereas the control calves had seroconverted to the K99

antigen. Seroconversion after ETEC infection has been recorded previously (Ellens, de Leeuw and Rozemond, 1979). The actively acquired K99 antibody was still detectable in the control calves 7 months post infection. It is possible that actively acquired antibodies were present in the sera of calves from all 3 vaccinated groups, but masked by the presence of passively acquired maternal antibody. Passively acquired antibody may also have inhibited the production of the calves own K99 antibodies as observed by Uhr and Baumann (1961) for other antigens.

It is uncertain from these studies which class of milk immunoglobulin provided protection of the calf against ETEC. The work in Chapter 4, Section A suggested that the prolonged milk antibody response was due to anti-K99 IgG, although IgA and IgM responses may also have occurred in the colostrum whey of oil adjuvant vaccinated cows. The results shown in Figures 4.4 and 4.5 are similar to those found by Snodgrass, Fahey, Campbell and Whitelaw (1980) with a rotavirus vaccine; prolonged, raised IgG₁ antibodies to rotavirus were found in the milk of vaccinated cows. Newby and Bourne (1976) have shown that IgG₁ is selectively transferred from the serum to colostrum in cows. It is probable therefore, that the raised IgG levels found in colostrum and milk in the present study were due largely to IgG₁, but further studies using specific monoclonal antibodies to IgG₁, IgG₂, IgA and IgM are clearly desirable to provide information on the effective immunoglobulins involved in the immune exclusion of ETEC.

The PHA assay confirmed the results of the ELISA and provided a comparative analysis of the antibody results. An advantage of

the ELISA as compared with the PHA test is that many samples can be tested at the same time without serial dilution and all results are related to one standard serum.

The antibody response to the rotavirus component of the vaccine was similar to that reported by Snodgrass, Fahey, Wells, Campbell and Whitelaw (1980) indicating that inclusion of the K99 antigen in the vaccine did not modify the immune response to the rotavirus component.

The clinical response of control calves to challenge by ETEC indicated that these animals were susceptible, although all but one of the calves survived, and their clinical signs were not as severe as the challenge experiment calves. Control calves sucked colostrum from their dams throughout the whole experimental period whereas experimental challenge calves initially received colostrum from their dams but were then given milk replacer; hence colostrum from unvaccinated mothers might therefore have provided some non-specific protection. Acres, Isaacson, Babuik and Kapitany (1979) used a ten-fold higher challenge dose than in these studies, and obtained much more severe effects with 9 of 10 calves dying and an average body weight loss over 24 hours of 14% compared with 1 of 6 deaths and a mean weight loss of 2.6% in Experiment 4. Thus the challenge dose could have been increased, although this may have obliterated the protective effect of the vaccine, a possibility pointed out by Myers (1976a).

Faecal excretion of ETEC over the 6 days post challenge was significantly reduced in calves from vaccinated cows compared with control calves. This finding may have epidemiological significance

under field conditions, as the vaccine should contribute to the reduction of environmental contamination by ETEC. Of the two methods used for identifying ETEC isolates, B44 serological identification was significantly higher than the K99 antigen dependent method. The possibility that K99⁻ B44 organisms were excreted was not investigated. In a vaccine trial conducted by Nagy (1980), in which the challenge strain was B41, faecal isolates were tested using anti-B41 serum and adsorbed K99 antiserum. Only 40 to 60% of B41 E. coli were K99⁺, and faecal excretion of these K99⁺ strains was not significantly reduced in the vaccinated groups. However Linggood and Porter (1980) reviewed a situation in pigs in which an in vivo 'plasmid curing effect' was observed. Piglets born to sows vaccinated with a heat inactivated E. coli vaccine which included K88⁺ E. coli ('Intagen', Unilever) and challenged with a K88⁺ E. coli strain were found to excrete K88⁻ variants of the challenge strain. The 'curing' or loss of the plasmid coding for K88 was postulated to have been due to factors present in the colostrum of immunised sows not associated with antibody activity against O or K88 antigens.

Two other recent studies have used K99 antigen extracts to immunise cows for passive protection of calves against K99⁺ ETEC infections (Acres, Isaacson, Babuik and Kapitany, 1979; Nagy, 1980). Both studies used extracts from the K12:K99 E. coli strain. It was concluded that high colostral K99 titres protected calves against challenge by B44 (Acres, Isaacson, Babuik and Kapitany, 1979) and B41 (Nagy, 1980). In these studies K99 antigen was the only known common antigen between the challenge strains and the vaccine extracts.

B41 and B44 strains possess the F41 antigen but the K12:K99 strain lacks this pilus antigen (de Graaf and Roorda, 1982; Morris, Thorns, Scott, Sojka and Wells, 1982). The K99 extract used in our vaccine was prepared from the B41 strain and contains K99, F41 and possibly O101 antigens (N. Chanter, personal communication). Since the vaccine (B41) and challenge (B44) strains used in Experiment 4 share both K99 and F41 antigens, antibodies against either of these may have been protective, a situation considered by Morris, Wray and Sojka (1980). The K30 antigen on strain B44 has also been reported to have adhesive properties (Smith and Huggins, 1978) from studies using B44 mutants. It is possible that the F41 antigen may have been responsible for adherence of K99⁻ B44 in the study cited, but no conclusive evidence is available to confirm this. Also pregnant cows vaccinated with O9:K30 strain passively protected calves against challenge by an O9:K30:K99 strain (Myers, 1978b). The antibody response to O101 antigen was not evaluated in this study, but antibodies to F41 antigen were detected in pooled colostrum whey from both oil vaccinated cows but not Alhydrogel vaccinated and unvaccinated cows (N. Chanter, personal communication).

This is the first recorded study in which a K99/rotavirus vaccine has been used to passively immunise calves against infection by K99⁺ ETEC and rotavirus (Snodgrass, Nagy, Sherwood and Campbell, 1982). Previous studies have used vaccines which comprised whole cell bacterins of ETEC, with no rotavirus component (Myers, Newman, Wilson and Catlin, 1973; Newman, Myers, Firehammer and Catlin, 1973; Myers, 1976a,b; Bagley and Call, 1979; Acres, Isaacson, Babuik and Kapitany, 1979; Acres, Forman and Kapitany, 1982). Although one report (Acres and Radostits, 1976) used an E. coli bacterin in

combination with oral administration of a modified live reo-virus-like vaccine to newly born calves. Varying degrees of success in the protection against K99⁺ ETEC infections were obtained in the cited works. In view of the possible role of K polysaccharide antigens in colonisation of the small intestine (Hadad and Gyles, 1982b) it would seem wise to include K polysaccharide antigens in a vaccine rather than depending on one common antigen such as K99. This line of research was considered by Myers (1980) when using a 4 strain bacterin.

The work in this Chapter has concentrated on the active immunisation of cows and the consequent passive protection of their calves through increased levels of effective antibody in colostrum and milk. Other methods which have been adopted include blocking attachment by inclusion of inert antigen in feed as carried out with K88 antigen in piglets (Davidson and Hirsh, 1976), or feeding an extract rich in K99 antibodies (Trainin, Brenner and Merion, 1980). Other practical approaches might involve continued feeding of bucket reared dairy calves with colostrum from vaccinated cows (Snodgrass, Stewart, Taylor, Krautil and Smith, 1982).

The pathogenic effects of ETEC could be controlled by neutralisation of enterotoxin. For calf ETEC this is difficult as the enterotoxin produced by the majority of strains, STa, is only weakly immunogenic. Myers, Newman, Wilson and Catlin (1973) used a crude toxin preparation from strain B44 which was effective only if vaccination was within 1 month of parturition. It is possible that their vaccine was effective because it contained low concentrations of O, K and K99 antigens.

Clearly there are many alternative methods available to immunise calves against ETEC diarrhoea but many must depend on the presence of specific antibodies in the gut lumen of newborn calves preventing colonisation by pathogenic ETEC and diarrhoea caused by these agents.

CHAPTER 5 SECTION A
EXPERIMENTAL CRYPTOSPORIDIUM SP INFECTIONS IN
LABORATORY MICE

INTRODUCTION

Cryptosporidium sp infections were first described in tamed wild mice by Tyzzer (1907, 1912) and subsequently in C57 mice by Hampton and Rosario (1966).

Studies using Cryptosporidium sp isolated from guinea pigs (Vetterling, Jervis, Merrill and Sprinz, 1971) suggested that Cryptosporidium sp were species specific, but recently subclinical infections were established in 1-day-old specific pathogen free (SPF) suckling mice and rats inoculated with Cryptosporidium sp infected faeces taken from calves, lambs or humans (Tzipori, Angus, Campbell and Gray, 1980).

During experimental infection studies using Cryptosporidium sp in calves and lambs (Chapter 5, Section B; Chapter 6, Sections A and B) it became apparent that there was a problem in assessing the infectious dose of an inoculum and the ability to maintain the viability of Cryptosporidium sp in stored inocula. This section of Chapter 5 describes the establishment of a laboratory system in which infective doses can be quantified and the viability of faeces containing Cryptosporidium sp can be monitored when stored under a variety of conditions. Other aspects of Cryptosporidium sp infections in mice were examined, such as; mouse strain susceptibility, susceptibility of mice of different ages and the effect of cyclophosphamide treatment.

Only formalin and household ammonia have so far been shown to inactivate faecal forms of Cryptosporidium sp (Campbell, Tzipori, Hutchison and Angus, 1982) thus there is a need to find a satisfactory (non-noxious) disinfectant for use in farm premises and laboratories. An investigation was therefore undertaken, using the laboratory mouse system, to examine the ability of 2 aldehyde based disinfectants to inactivate faecal Cryptosporidium sp.

MATERIALS AND METHODS

Animals

The mice used were produced under SPF conditions. Suckling mice were kept with their mothers for the duration of the experiment and all mice were maintained in plastic isolators after transfer from the SPF isolation unit. In all, two random bred strains of mice, Schneider Swiss white and Porton, and six inbred strains, CBA, C57 black, Balb/c, athymic CBA-nude (nu/nu), Porton (derived from the random bred stock) and Hairless (HR/HR-ADR) were used, but unless stated otherwise routine experiments employed the inbred Porton strain of mouse.

The pathogenicity of passaged Cryptosporidium sp was tested in two gnotobiotic lambs derived and maintained as described in Chapter 6.

Administration of inocula to mice

Young mice were inoculated by the oral route with 0.1ml of faecal suspension or gut homogenate using a 23 gauge needle tipped with plastic tubing. The dose was increased to 0.2ml for mice which were 21 days or older.

Preparation of inocula

Cryptosporidium sp present in the faeces of a diarrhoeic calf (Tzipori, Campbell, Sherwood, Snodgrass and Whitelaw, 1980) was passaged once in SPF rats and twice in SPF lambs (Tzipori, Angus, Gray, Campbell and Allan, 1981) and then in SPF mice to produce inocula 1 to 4 (Figure 5.1). All inocula were approximately 20% homogenates of faeces (v/v) (inoculum 1) or whole gut (w/v) (inocula 2 to 4) in 5% BSA.

The faecal preparations (inocula 5 and 6) used in Experiment 5 contained a calf Cryptosporidium sp isolate that had been passaged 4 times in gnotobiotic lambs (Dr. D.R. Snodgrass, unpublished results). Inoculum 5 was bacteria and fungal free following in vivo and in vitro antibiotic treatment with gentamicin, ampicillin, streptomycin, oxytetracycline and nystatin and inoculum 6 contained Cryptosporidium sp and 2 strains of non-enterotoxigenic E. coli, a Bacillus spp, and Streptococcus faecalis isolated from the faeces of a normal 7-day-old calf.

Detection of Cryptosporidium sp

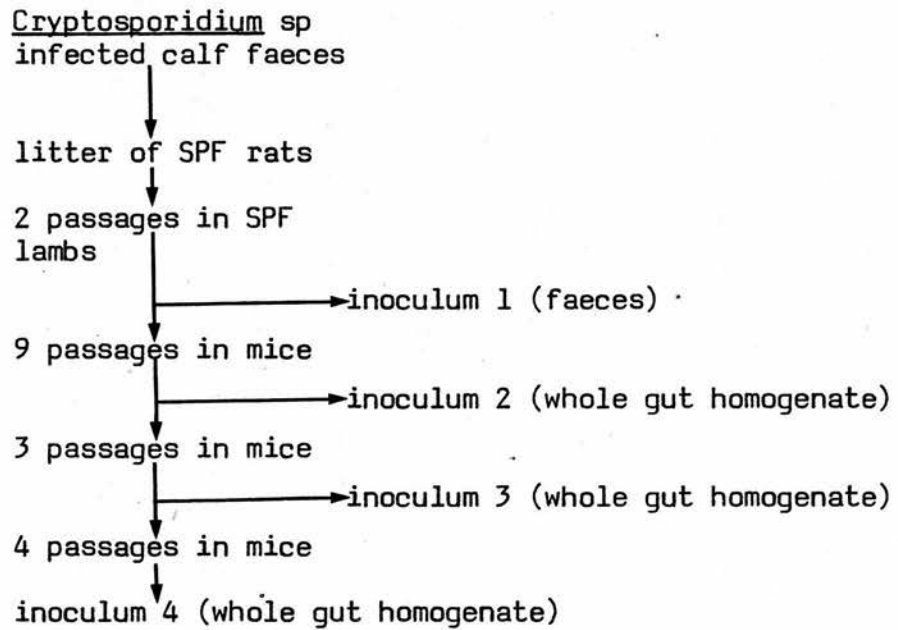
Smears of caecal contents were stained by Giemsa's method as described in Chapter 2. Segments of intestine 0.5cm long from the duodenum, terminal ileum and caecum were taken into 10% buffered formol saline or Carnoy's fixative. Paraffin sections 5µm thick were stained by Mayer's haematoxylin and eosin (HE).

EXPERIMENT 1: SUSCEPTIBILITY OF MOUSE STRAINS TO INFECTION BY CRYPTOSPORIDIUM SP

METHODS

Two litters each of 8 strains of mice were inoculated at 1 to

Figure 5.1 Sequential passage of a calf *Cryptosporidium* sp isolate



4 days of age with inoculum 1, 2 or 3 (Table 5.1). Mice were killed and examined daily from 2 to 7 days post inoculation (p.i.) for evidence of infection. Two uninoculated litters of inbred Porton mice were placed in the same isolators as inoculated mice.

RESULTS

All 8 strains of mice inoculated between 1 and 4 days of age were susceptible to Cryptosporidium sp infection (Table 5.1), although none of the mice were clinically ill at any time. Oocysts were detected in the faeces from 3 to 7 days p.i.

There was no evidence of infection with Cryptosporidium sp as determined by histological and faecal examination of uninoculated age-matched mice from the same isolators as inoculated mice (Figure 5.3a).

Histological examination of infected mice demonstrated Cryptosporidium sp in large numbers on the villi of the terminal ileum and the luminal surface of the caecum (Figures 5.3b and 5.3c), but only occasionally in the duodenum. Significant pathological changes were not observed at any intestinal site.

EXPERIMENT 2: RELATIONSHIP OF AGE TO SUSCEPTIBILITY TO INFECTION BY CRYPTOSPORIDIUM SP

METHODS

Inoculum 2 was administered to 24 suckling mice from 4 litters at 4 days of age, and inoculum 3 to 24 weaned mice at 21 days of age. Two mice from each group were killed and examined for infection on 11 occasions between 2 and 16 days p.i. In addition 21 to 42-day-old mice of the Hairless, Swiss White and CBA-nude strains were given inoculum 1 and one mouse per strain examined daily for evidence of infection for 14 days p.i.

Table 5.1 Results of exposure of strains of mice to *Cryptosporidium* sp at 1-4 days

| Mouse strain ^a | Inoculum | Infection established |
|---------------------------|----------|-----------------------|
| Random bred Porton | 1 | + |
| Schneider Swiss White | 1 | + |
| Inbred Porton | 1 | + |
| Balb/c | 2 | + |
| CBA-nude | 3 | + |
| CBA | 3 | + |
| Hairless (HR/HR-ADR) | 3 | + |
| C57 black | 3 | + |
| Control mice | None | - |

a; at least 6 mice exposed from each litter.

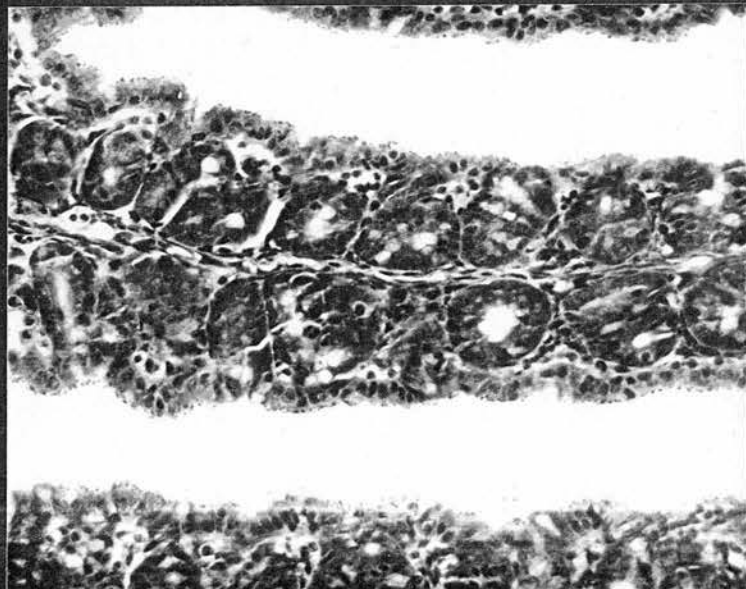
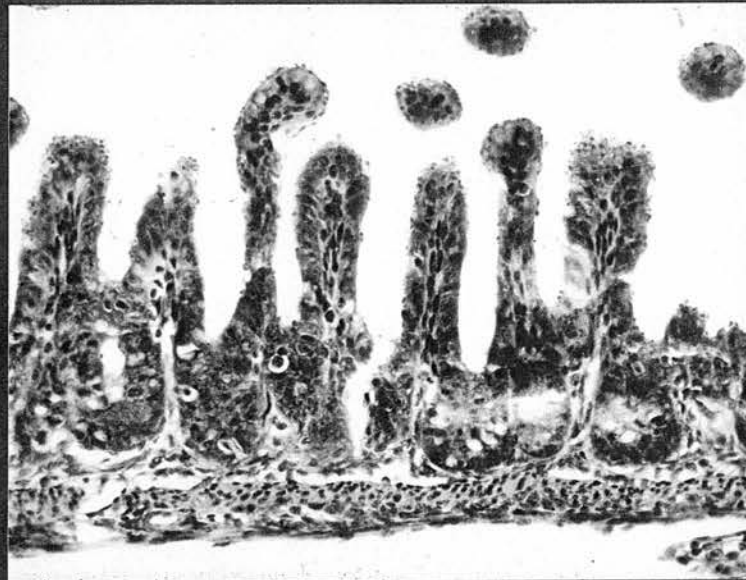
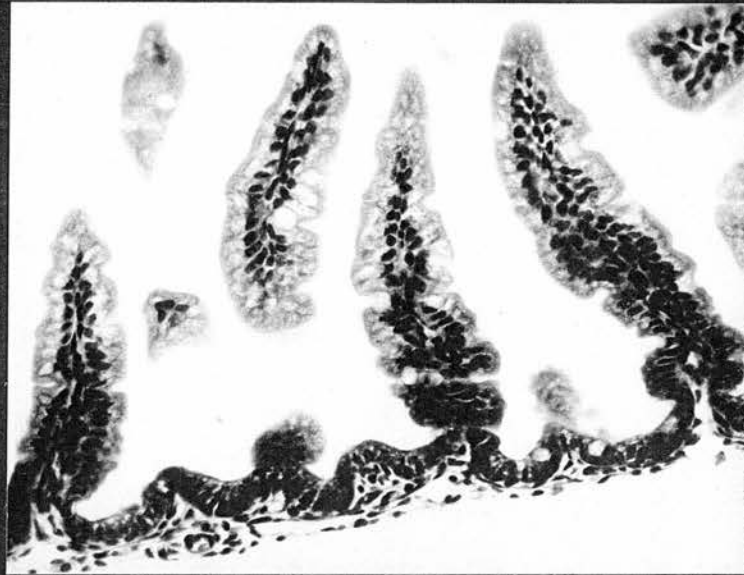
++; *Cryptosporidium* sp detected by histology and faecal examination.

-; no *Cryptosporidium* sp detected.

Figure 5.3a Ileum of an uninfected 1-week-old inbred Porton mouse. (Haematoxylin and eosin (HE), x 370)

Figure 5.3b Ileum of a 1-week-old inbred Porton mouse infected with Cryptosporidium sp. Endogenous stages can be seen along the sides and on the tips of villi. (HE x 370)

Figure 5.3c Caecum of a 1-week-old inbred Porton mouse infected with Cryptosporidium sp. Organisms can be seen on the luminal surface of the mucosa. (HE, x 370)



RESULTS

The mucosal surface of the ileum was heavily infected at 5 to 12 days p.i. in mice inoculated at 4 days of age, but light levels of infection were only detected between 7 to 11 days p.i. in mice inoculated at 21 days of age (Table 5.2). Histological and faecal examination techniques correlated well, although histology was the more sensitive and detected the continuing presence of infection after organisms were no longer observable in the faeces.

None of the 28 Hairless and Swiss White mice inoculated at 21 to 42 days became infected and only 1 of the 14 CBA-nude mice showed evidence of infection at 9 days p.i.

EXPERIMENT 3: (a) EXAMINATION OF THE IMMUNOSUPPRESSIVE EFFECTS OF CYCLOPHOSPHAMIDE

METHODS

One of two groups of 10 mice (Group 1, Table 5.3) 21 days of age was treated intraperitoneally (i.p.) with cyclophosphamide at 70mg/kg (0.1ml of 15mg/ml in sterile distilled water) on two occasions 7 days apart (Berenbaum and Brown, 1964). The immunosuppressive activity of cyclophosphamide was evaluated by assaying the serological response to 0.5ml of louping-ill virus vaccine (Moredun Type Vaccine, Wellcome) injected i.p. in both groups at 23 days of age. All mice were bled at 42 days old and serum antibody titres to louping-ill virus were measured by the haemagglutination inhibition test (HAI) by Dr. B. Shaw (Moredun Research Institute) using the method of Clarke and Casals (1958).

RESULTS

The reciprocal HAI titres to louping-ill virus vaccine in cyclophosphamide treated mice (Group 1) was 10 to 20, with reciprocal

Table 5.2 Age related susceptibility of mice to infection by *Cryptosporidium* sp

| Age at inoculation (days) | Demonstration of infection, days post inoculation | | | | | | | | | | | |
|------------------------------|---|----|-----|-----|-----|-----|-----|----|----|----|----|--|
| | 3 | 5 | 6 | 7 | 8 | 10 | 11 | 12 | 13 | 14 | 16 | |
| 4 | - | ++ | +++ | +++ | +++ | +++ | +++ | ++ | + | + | + | |
| 21 | - | - | NT | + | + | - | + | - | - | - | NT | |

++; mild infection detected by histological examination only.

++; mild infection indicated by histology and faecal shedding of *Cryptosporidium* sp.

+++; moderate to heavy infection indicated by histology and faecal shedding of *Cryptosporidium* sp.

-; no infection detected by either histological or faecal examination.

NT; not tested.

Table 5.3 Experimental protocol for mice treated with
cyclophosphamide and louping-ill virus vaccine

| Group | Number of mice | cyclophosphamide ^a (70mg/kg) | louping-ill ^a virus vaccine (0.5ml) | Age when bled (days) |
|-------|-------------------|--|--|----------------------------|
| 1 | 10 | 21,28 | 23 | 42 |
| 2 | 10 | not treated | 23 | 42 |

a; age of mice at inoculation (days).

titres of 160 to 320 in the untreated mice (Group 2).

EXPERIMENT 3: (b) INFECTION OF CYCLOPHOSPHAMIDE TREATED MICE WITH CRYPTOSPORIDIUM SP

METHODS

Four groups each comprising eight, 21-day-old mice were treated with cyclophosphamide and louping-ill virus vaccine (Table 5.4). Mice in groups 1, 2 and 4 were inoculated with Cryptosporidium sp inoculum 4 two days after the second cyclophosphamide injection. Pairs of mice from each group were killed daily on days 3 to 8 p.i. and examined for Cryptosporidium sp infection and serum antibody titres to louping-ill virus vaccine.

RESULTS

The antibody response to louping-ill virus vaccine was again reduced from 160-320 in untreated mice (Group 2, Table 5.4) to 10-40 in cyclophosphamide treated mice (Groups 1 and 3, Table 5.4). However, no mice in any group became infected with Cryptosporidium sp.

EXPERIMENT 4: EFFECT OF MULTIPLE PASSAGE OF CRYPTOSPORIDIUM SP IN MICE ON PATHOGENICITY FOR LAMBS

INTRODUCTION

The aim of this experiment was to examine whether the calf Cryptosporidium sp originally shown to be pathogenic for SPF lambs (Tzipori, Angus, Gray, Campbell and Allan, 1981) remained pathogenic for lambs after 12 passages in suckling mice.

METHODS

Two, 2-day-old gnotobiotic lambs were orally inoculated with 5ml of the twelfth mouse passage of Cryptosporidium sp (inoculum 3).

Lambs were monitored daily for clinical signs of infection and faecal shedding of the organism. Intestinal tissues were taken under terminal anaesthesia from one lamb at 5 days and the other at 11 days p.i. for histological examination (Chapter 2).

RESULTS

Both gnotobiotic lambs developed diarrhoea, became anorectic and dehydrated. Histological examination of the intestines of these lambs gave findings which were consistent with those obtained previously (Chapter 6, Section A), namely villous atrophy, fusion and epithelial cross-bridging in the distal small intestine. Large numbers of Cryptosporidium sp were apparent in the ileum, with moderate infection present at other intestinal sites. The caecum of the lamb killed at 5 days p.i. was infected, but no pathological changes were apparent at this site.

EXPERIMENT 5: LABORATORY STORAGE OF CRYPTOSPORIDIUM SP

METHODS

Storage

The effect of freezing and freeze drying of inoculum 3 in the presence of cryopreservatives under various conditions was investigated. All cryopreservatives listed in Table 5.5 were prepared in PBS and mixed with an equal volume of inoculum 3.

In addition, stability of Cryptosporidium sp in inocula 5 and 6 stored up to 6 months at 4 °C in distilled water, 5% BSA, PBS and 2.5% potassium dichromate was studied. Cryptosporidium sp were also stored at room temperature and 37 °C in PBS.

Titration of Cryptosporidium sp

The stored preparations were titrated periodically in mice

Table 5.4 Experimental protocol for mice treated with cyclophosphamide, immunised with louping-ill virus vaccine and inoculated with Cryptosporidium sp

| Group ^a | Age in days for | | | mice killed |
|--------------------|--------------------------------------|---------------------------------------|---|-------------|
| | cyclophosphamide treatment (70mg/kg) | louping-ill virus vaccination (0.5ml) | Cryptosporidium sp administration (0.2ml) | |
| 1 | 21,28 | 23 | 35 | 38-43 |
| 2 | NT | 23 | 35 | 38-43 |
| 3 | 21,28 | 23 | NT | 38-43 |
| 4 | NT | NT | 35 | 38-43 |

a; 8 mice per group.

NT; not tested.

Table 5.5 Freezing and freeze drying methods used to store
Cryptosporidium sp

| Cryopreservative | Storage Conditions |
|---|---|
| 1. 5% PVP | immersion in liquid |
| 2. 5% Glycerol | nitrogen for 10 minutes |
| 3. 10% DMSO | then thaw at 37 °C |
| 4. 10% DMSO and 20% BSA | |
| 5. SPGA and DMSO to 8% (v/v) | immersion in liquid nitrogen then thaw at RT |
| 6. 20% NRS and 10% Glycerol (equilibrated at 15-20 °C for 20 hours) | cool to -70 °C then thaw at RT |
| 7. 10% DMSO | -20 °C for 3 days, thawed at RT |
| 8. 20% Glycerol | -20 °C for 3 days, thawed at RT |
| 9. PBS | -20 °C for 14 days, thawed at RT |
| 10. PBS | -70 °C for 14 days, thawed at RT |
| 11. Freeze drying in 10% Glycerol | 2 days at 4 °C |

PVP; Poly-vinyl-pyrrolidone (Mol. Wt. 44,000).

DMSO; Dimethyl sulphoxide.

SPGA; Sucrose 74.6 g/litre, KH_2PO_4 1.25 g/litre,
L-glutaric acid 0.91 g/litre, and BSA 10 g/litre.

NRS; normal rabbit serum.

RT; room temperature.

(Appendix 3.). Serial $10^{0.5}$ dilutions of stored preparations were made in PBS and 1 litter (average 6 mice) of 1 to 4-day-old mice was inoculated with each dilution. Mice were killed at 6 days p.i. and the Giemsa stained smears of caecal contents from each mouse examined for Cryptosporidium sp. The 50% mouse infective dose (MID_{50}) was calculated using the method of Reed and Muench (1938). Two selected titrations were used to compare Giemsa staining of faeces and histology as methods for the detection of infected animals.

RESULTS

Freezing destroyed the activity of Cryptosporidium sp irrespective of the cryopreservative method used.

There was a progressive loss of infectivity of both inocula 5 and 6 when held at $4^{\circ}C$ irrespective of the diluent (Figures 5.2a and 5.2b). No infectivity was detectable in inoculum 6 after 2 months storage in distilled water. The most stable preparations were those maintained in 2.5% potassium dichromate, in which infectivity lasted at least 6 months. Complete loss of infectivity of inoculum 6 in PBS occurred within 2 weeks at room temperature and within 5 days at $37^{\circ}C$. However inoculum 5 maintained infectivity for least 4 weeks at room temperature.

A comparison of Giemsa staining of faeces and histology for detection of infected animals utilised inoculum 5 after 56 days storage at $4^{\circ}C$ in 2.5% potassium dichromate and inoculum 6 after 58 days storage at $4^{\circ}C$ in 5% BSA. Inoculum 5 yielded titres of $10^{3.8}$ and $10^{4.25}$ MID_{50}/ml by Giemsa staining and histological examination respectively, whilst the titres of inoculum 6 were $10^{2.37}$ and $10^{3.25}$ MID_{50}/ml respectively.

Figure 5.2a Infectivity of inoculum 5 for suckling mice after laboratory storage.

- — ■ Storage in PBS at 4 °C.
- △ — △ Storage in 2.5% potassium dichromate at 4 °C.
- — □ Storage in PBS at room temperature.

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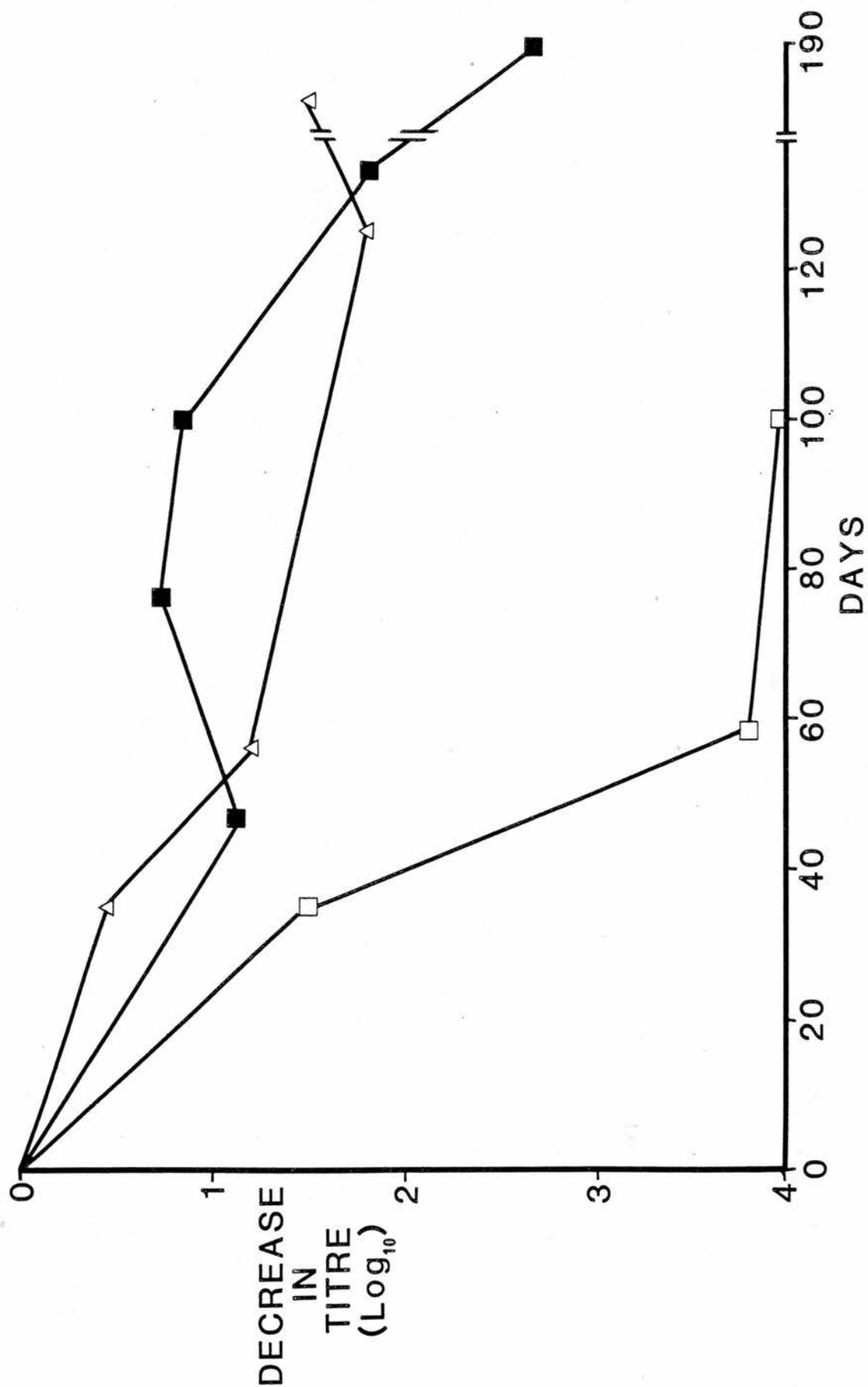
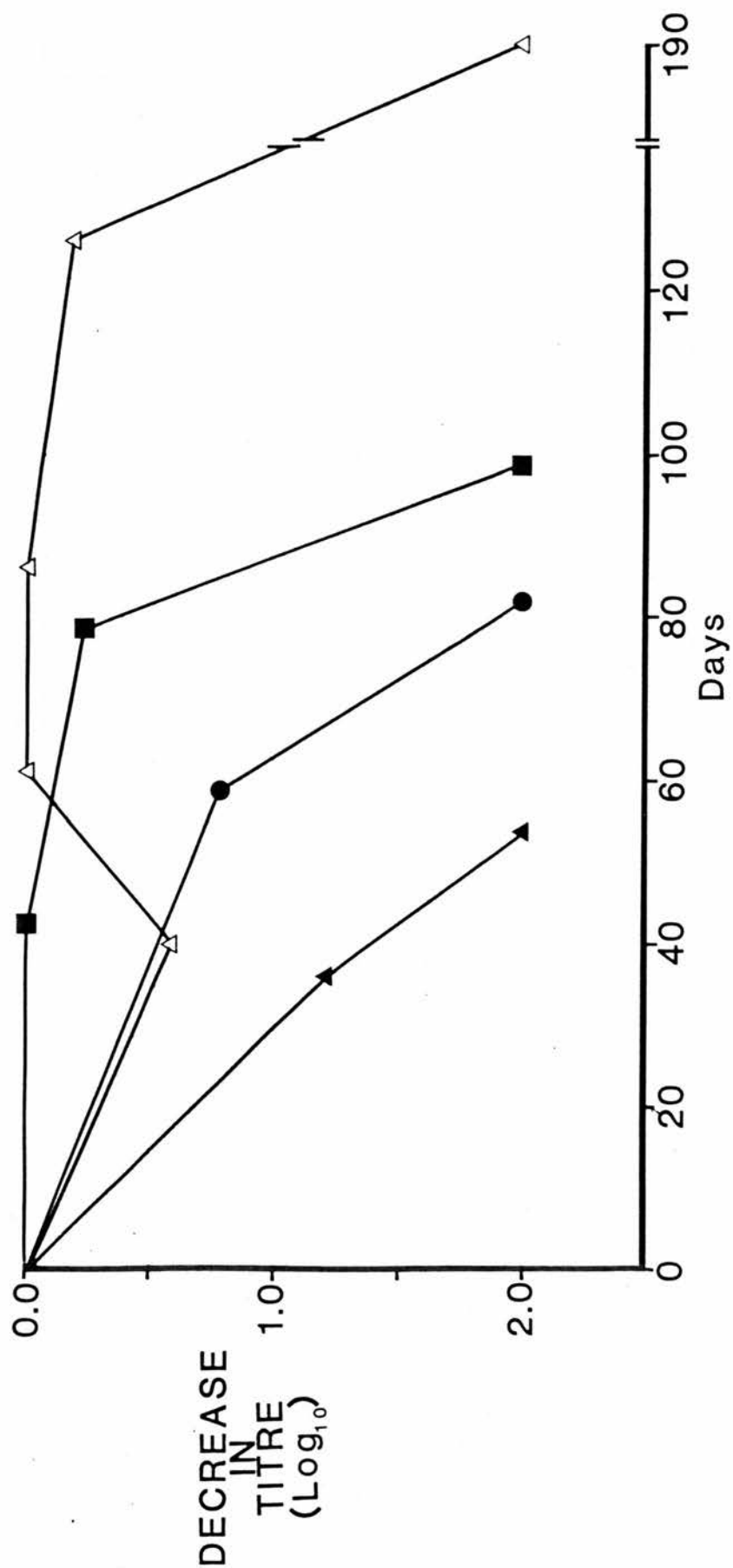


Figure 5.2b Infectivity of inoculum 6 for suckling mice after laboratory storage .

- — ■ Storage in PBS at 4 °C.
- △ — △ Storage in 2.5% potassium dichromate at 4 °C.
- — ● Storage in 5% BSA at 4 °C.
- ▲ — ▲ Storage in distilled water at 4 °C.



EXPERIMENT 6: THE EFFECT OF TWO ALDEHYDE-BASED DISINFECTANTS ON THE
INFECTIVITY OF CRYPTOSPORIDIUM SP IN MICE

METHODS

Disinfectants

The disinfectants tested were Tegodor (Th. Goldshmidt, A.G., Chemische Fabrikien, Desinfektion Postfach. 17, 4300 Essen) and Formula-H (Hoechst (UK) Ltd., Walton Manor, Walton, Milton Keynes, Bucks.). Tegodor concentrate contained cetylkonium chloride; 3% (w/v) benzylkonium chloride; 10% glutaraldehyde (50% w/v) and 20% formaldehyde (37% w/v). Formula-H concentrate contained 0.129% (w/v) tris-n-butyl benzoate; 4.6% (v/v) formaldehyde (37%) and 1.33% (v/v) isopropyl alcohol.

Exposure of Cryptosporidium sp to disinfectants

Equal volumes of inoculum 4 and disinfectants diluted in distilled water were maintained at room temperature as follows; 6% Tegodor and 17% and 40% Formula-H for 1 hour; 0.1% and 3.0% Tegodor and 3.5% Formula-H for 1 hour and 6 hours. Solutions of 6% Tegodor and 40% Formula-H were toxic for mice, so after treatment with these concentrations of disinfectants, inoculum 4 was centrifuged at 850g at room temperature for 10 minutes and the deposit resuspended in distilled water.

One litter of 1-4-day-old mice was inoculated with each disinfectant treated faeces, with control litters receiving inoculum 4 in distilled water, or distilled water only. Pairs of mice were killed at 5 and 7 days p.i. and the remaining mice were killed 12 days p.i.

RESULTS

The results are shown in Table 5.6. Neither Tegodor nor Formula-H

Table 5.6 Infectivity of *Cryptosporidium* sp for 1 to 4-day-old mice after treatment with two aldehyde-based disinfectants

| Exposure and time of treatment of inoculum 4 with disinfectant | Infection of mice after inoculation with <i>Cryptosporidium</i> sp treated with various disinfectants | | | | | | | |
|--|---|-----|-----|-------|------------------------|--------------------|-------|--|
| | Tegodor ^a | | | | Formula H ^a | | | |
| | 0.1 | 1.0 | 3.0 | 6.0 | 8.5 | 17.0 | 40.0 | |
| 1 hour at RT | + | + | + | toxic | + | toxic ⁺ | toxic | |
| 1 hour ^b at RT | ND | ND | ND | + | ND | ND | + | |
| 6 hours at RT | + | + | + | ND | + | ND | ND | |

++; histological and faecal detection of *Cryptosporidium* sp.

toxic; toxic to mice at the concentration of disinfectant used.

ND; not done.

RT; room temperature.

a; concentration of disinfectant in distilled water (%).

b; organisms centrifuged and resuspended in distilled water after treatment.

were able to destroy the infectivity of faecal Cryptosporidium sp under any experimental condition.

DISCUSSION

These experiments demonstrated that Cryptosporidium sp sub-clinically infected 8 different strains of 1 to 4-day-old SPF mice, but at 21 days of age or older the same strains of mice had a lower susceptibility to infection. This age related susceptibility has been recognised by other workers (Tzipori, Angus, Campbell and Gray, 1980; Heine and Boch, 1981) and could explain why Cryptosporidium sp isolated from guinea pigs (Vetterling, Jervis, Merrill and Sprinz, 1971) and cats (Iseki; 1979) failed to infect weanling mice or 7-week-old ICR strain mice respectively. However, work by Tyzzer (1907; 1912) who used newly weaned tamed mice, suggested that infection by two species of Cryptosporidium sp (C. muris and C. parvum) was not age dependent.

In view of the number of organisms present in the caecum of 1 to 4-day-old mice, it was surprising that no pathological changes were apparent, and that the infection remained subclinical. However these results agree with those of other workers (Tyzzer, 1910; 1912; Hampton and Rosario, 1966). Only severe infection by C. muris (Tyzzer, 1910) led to dilatation of gastric glands and leucocyte infiltration. This situation is in contrast to the severe clinical disease and pathological changes observed in young lambs experimentally infected with the organism (Chapter 6, Section A).

Cryptosporidium sp infections have been observed in immunosuppressed humans (Meisel, Perera, Meligro and Rubin, 1976; Clinico-pathology Conference, 1980; Weisburger, Hutcheon, Yardley, Roche, Hillis

and Charache, 1980) and in immunologically normal humans (Tzipori, Angus, Gray and Campbell, 1980; Reese, Current, Ernst and Bailey, 1982). In the present experiments, cyclophosphamide was demonstrated to impair the antibody response of mice to a viral antigen. The reduction in antibody response to viral antigen served as an indicator of general antibody depression as cyclophosphamide has been shown to affect B-lymphocyte production (Stockman, Heim, South and Trenlin, 1973). This effect apparently failed to increase the susceptibility of weanling mice to Cryptosporidium sp infections. The continued resistance to infection in the light of generally depressed antibody response may have been due to cell mediated reactions which were not monitored or factors at the mucosal surface such as mucus exclusion. All but one of the athymic CBA-nude mice inoculated at 28 days of age were apparently not susceptible to Cryptosporidium sp infection, again the cell mediated response or antibody response were not measured in these animals.

The calf Cryptosporidium sp isolate used was shown to retain its pathogenicity for gnotobiotic lambs after 12 passages in mice and the organism remained infectious for mice after 16 mouse passages. It is not known whether all Cryptosporidium sp isolates behave in this manner but Heine and Boch (1981) passaged a calf Cryptosporidium sp isolate in 3 day old NMRI mice, then successfully infected a goat with material from the 4th passage. A passage system in mice makes the maintenance of field isolates much easier and provides a 'biological filtration' system to remove enteric viruses such as rotavirus or coronavirus, and pathogenic bacteria, including enterotoxigenic E. coli.

Continuous biological passage is time consuming and costly, and there would be many advantages in being able to store isolates provided their pathogenicity can be maintained. The various cryo-preserved used failed to preserve the organism at low temperatures, although methods 5 and 6 (Table 5.5) have been successfully used for the long-term storage of rickettsiae (Bovarnick, Miller and Snyder, 1950) and Toxoplasma gondii (Eyles, Coleman and Cavanaugh, 1956) respectively. Storage of inoculum 6 at room temperature or 37 °C in PBS resulted in inactivation of Cryptosporidium sp within 2 weeks, but at 4 °C in PBS the organisms remained viable for 3-4 months. Heine and Boch (1981) also reported that calf faeces containing Cryptosporidium sp remained viable for 3-6 months when stored at 5 °C. They also showed that the organism was inactivated if heated at 50 °C for 15 minutes or stored at -18 °C for 24 hours. Furthermore, Pohlenz, Moon, Cheville and Bemrick, (1978) have reported that Cryptosporidium sp could be detected in faeces up to 45 days after storage at 4 °C. These findings are important epidemiologically in demonstrating that the organism may survive in faeces for a considerable period in a temperate climate.

In addition to being bacteria free, inoculum 5 contained a larger number of infective parasites than inoculum 6 (Figures 5.2a and 5.2b). It is possible that these two factors may have been responsible for the prolonged infectivity during storage of inoculum 5 compared with inoculum 6. Bacteria might reduce the viability of Cryptosporidium sp by the production of toxic products or by altering the character of the storage fluid.

The use of the mouse titration method to quantitate Cryptosporidium sp inocula was preferred to the flotation technique

described by Iseki (1979), the latter, in our experience gave only a small percentage of the organisms in faeces; moreover it is impossible to assess the viability of the parasite. Heine and Boch (1981) demonstrated that flotation methods distorted oocysts and hence affected their viability. With the mouse titration method an overall viability, measured as MID_{50} , is obtained for each inoculum so that comparisons between inocula can be made.

The sensitivity of the mouse titration method was improved by employing histological examination of the gut as an index of infection rather than relying solely on Giemsa staining of faecal smears. Both methods correlated well in the two titrations tested but the percentage of infected mice detected by histological examination was always higher.

The two aldehyde-based disinfectants proved to be ineffective in reducing the viability of Cryptosporidium sp (Angus, Sherwood, Hutchison and Campbell, 1982). Previous studies (Campbell, Tzipori, Hutchison and Angus, 1982) suggested that formalin and household ammonia reduced the infectivity of Cryptosporidium sp for mice, but both these reagents are unpleasant and hazardous to use because of their noxious nature. Tegodor was ineffective at twice the concentration recommended by the manufacturer, and Formula-H did not reduce the viability of Cryptosporidium sp at concentrations that have been reported by the manufacturer to destroy canine parvovirus, distemper and infectious hepatitis viruses, feline panleucopaenia virus and many pathogenic bacteria. Thus these two disinfectants offer no practical alternative to formalin or ammonia although many other disinfectants remain to be tested.

No effective therapy against Cryptosporidium sp infections is known at the present. Tzipori, Campbell and Angus, (1982) tested anticoccidial drugs, none of which eliminated experimental Cryptosporidium sp infections in suckling mice. Chemotherapeutic agents might have activity if given prophylactically and this requires to be tested.

The laboratory mouse is a very convenient model for the study of many aspects of Cryptosporidium sp infections, but large numbers of mice are needed for such studies. This problem could be obviated by the use of a tissue culture system, although the mouse model would still be required for studies on the immunology of Cryptosporidium sp infections and its life cycle. The tissue culture system could be utilised for the isolation and multiplication of the parasite and for use as a titration system.

In summary, the study has shown that various strains of laboratory mice developed subclinical infections with Cryptosporidium sp at 1 to 4 days of age, but only transient infections could be established at 21 days of age or older. Immunosuppression of 21-day-old mice with cyclophosphamide failed to render them more susceptible to infection. Laboratory storage conditions for Cryptosporidium sp were investigated and storage in PBS or 2.5% potassium dichromate at 4 °C was found to be successful for 4 to 6 months. The parasite could also be discontinuously passaged in mice without loss of its pathogenicity for gnotobiotic lambs, and a titration system in mice was developed which enabled quantitation of the infectivity of inocula used in experimental infection studies.

CHAPTER 5 SECTION BEXPERIMENTAL CRYPTOSPORIDIUM SP INFECTIONS IN CALVESINTRODUCTION

The aim of this study was to examine the effect of experimental infections with Cryptosporidium sp in conventionally reared and specific pathogen free (SPF) calves.

At the outset of this investigation information on experimental cryptosporidiosis in calves was available from two reports (Pohlenz, Moon, Bemrick and Cheville, 1978; Nagy, Antal and Lakner, 1980). Both these experimental infections were complicated by the presence of other enteropathogens so no clear cut conclusions could be made concerning the pathogenicity of Cryptosporidium sp per se in calves. This study aimed to examine the pathogenicity of Cryptosporidium sp in conventionally reared and SPF calves shown to be free of other enteropathogens.

MATERIALS AND METHODSAnimals

Four colostrum deprived, SPF Ayrshire bull calves (calves 1 to 4, Table 5.7) and 3 colostrum fed conventionally reared Jersey bull calves (calves 5 to 7, Table 5.7) were used. The SPF calves were caught at birth in sterile towels, then transferred through an iodophor bath into positive pressure plastic isolators for rearing. The colostrum fed calves were allowed to suckle their dam for at least 36 hours and then transferred from the farm at 48 hours to an isolation room at Moredun Research Institute. All calves were fed reconstituted milk as outlined in Chapter 6, Section B.

Table 5.7 Experimental design

| Calf number | Breed | Status | Age at inoculation with <u>Cryptosporidium</u> sp (days) | Age at necropsy (days) |
|-------------|-------|--------|--|------------------------|
| 1 | A | CD | 5 | 9 |
| 2 | A | CD | 5 | 13 |
| 3 | A | CD | 10 | 19 |
| 4 | A | CD | 10 | 22 |
| 5 | J | CF | 9 | 15 |
| 6 | J | CF | 9 | 15 |
| 7 | J | CF | 7 ^a | 24 |

A; Ayrshire.

J; Jersey.

CD; colostrum deprived.

CF; colostrum fed.

a; age of introduction to the contaminated area.

Cryptosporidium sp inoculum and inoculation of calves

The inoculum was derived from calf faeces which contained cryptosporidial oocysts but no other detectable enteric pathogens, in particular ETEC, rotavirus, coronavirus or Salmonella spp (see Chapter 3 for details of screening). Calves 1 to 6 were inoculated orally at times shown in Table 5.7 with 20ml of a 20% (v/v) faecal homogenate in 5% BSA. The occurrence of naturally acquired infection from a contaminated environment was tested by the introduction of calf 7 to the isolation room that had housed calf 6, 2 days previously.

Clinical observations

All calves were clinically examined twice daily throughout the experiment. Symptoms such as anorexia, faecal consistency and evidence of dehydration i.e. sunken eyes, tightness of skin, recumbency and coldness of extremities were noted. Due to the limitations of the isolation premises calves could not be weighed or their urine output monitored, faecal dry matters were not estimated.

Microbiology

Demonstration of Cryptosporidium sp excretion by Giemsa staining of faecal smears, and the screening of faecal swabs for ETEC, rotavirus, and enteric viruses were as described in Chapters 2 and 3.

Necropsy procedures

Calves were necropsied as described in Chapter 2 at the times indicated in Table 5.7.

Indirect immunofluorescence test for ETEC and rotavirus

Indirect immunofluorescence tests were performed as described in Chapter 2 using rabbit antisera against ETEC strain B44 and lamb rotavirus and sheep anti-rabbit IgG antiserum conjugated to fluorescein isothiocyanate. All antisera were diluted 1/20 in PBS.

Enzymology

Tissue from the upper jejunum, mid-gut and ileum without Peyer patches was examined for lactase activity as described in Chapter 6, Section A.

RESULTS

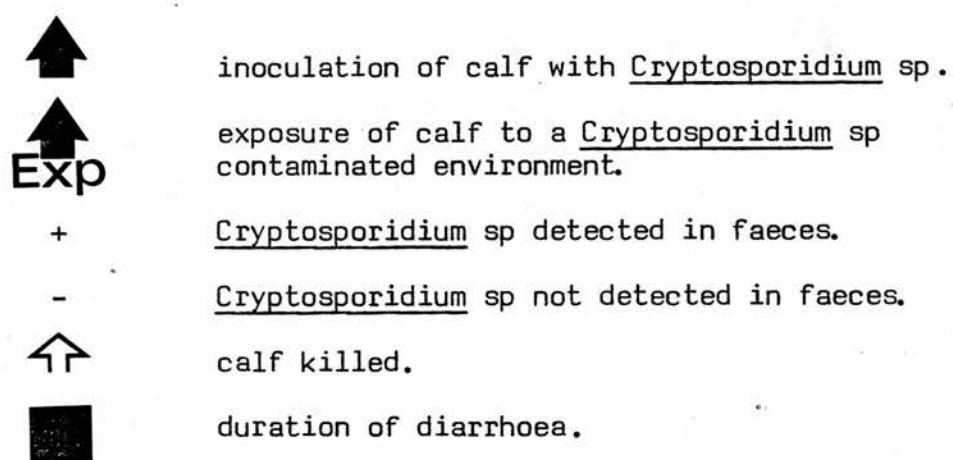
Microbiological and clinical observations

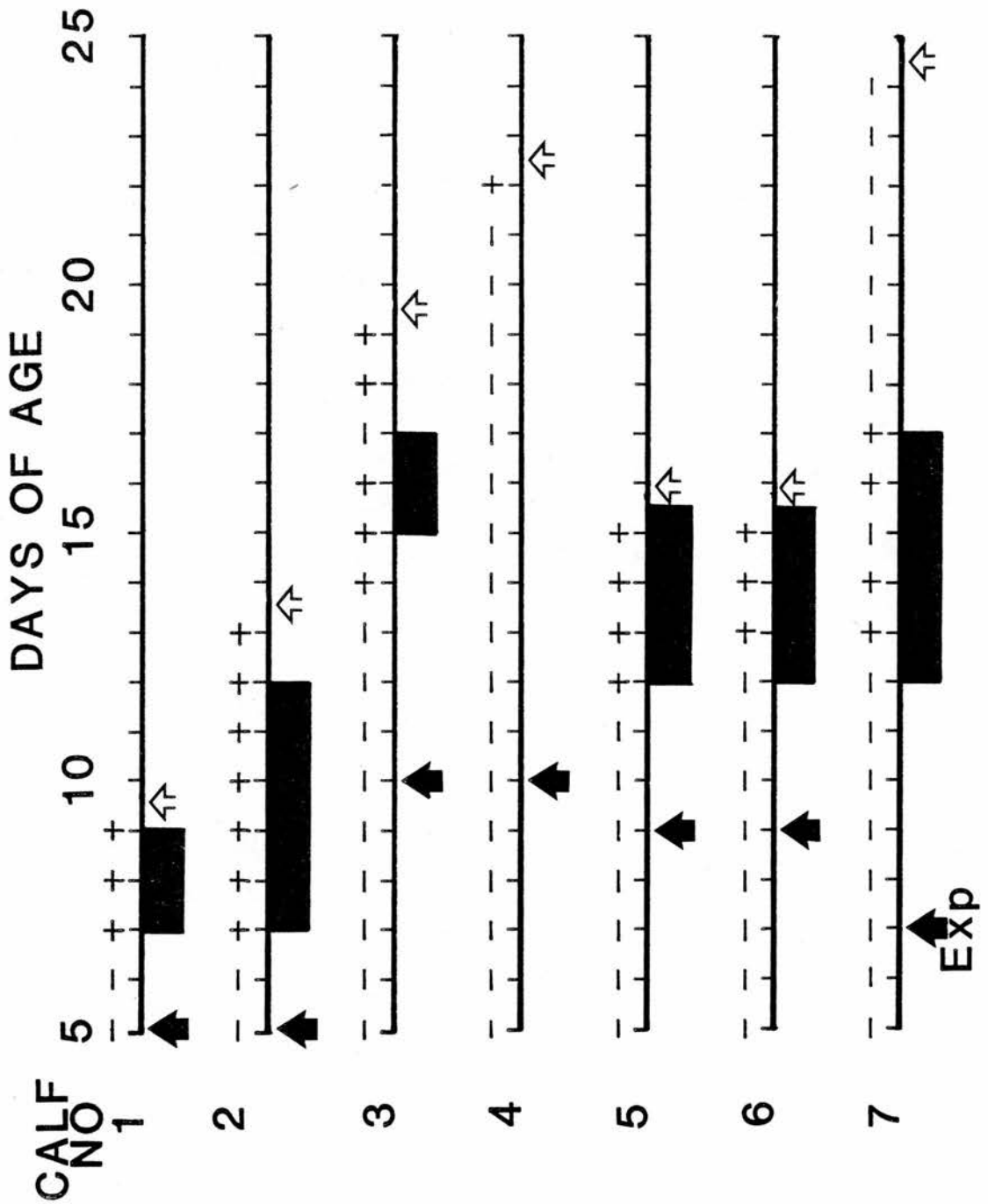
The Cryptosporidium sp inoculum was shown by periodic testing in suckling inbred Porton mice to remain viable for the whole 2 month experimental period. The inoculum was not titrated at any stage, although a decrease in titre was likely to have occurred (Section A of this chapter).

The faecal shedding of Cryptosporidium sp is illustrated in Figure 5.4. On some occasions during severe diarrhoea no organisms were detected in the faeces. At no time pre or post inoculation with Cryptosporidium sp were any other enteric pathogens detected. Cryostat sections of intestinal sites were found to be negative in the indirect immunofluorescence test for ETEC strain B44 and rotavirus infection.

All but one of the calves inoculated with Cryptosporidium sp first excreted organisms 2 to 4 days p.i. The exception, calf 4, was found to be infected at necropsy on day 12 p.i. Calf 7 which was indirectly exposed to Cryptosporidium sp first excreted the

Figure 5.4 Cryptosporidium sp infection of calves; clinical response and faecal shedding.





organism 6 days after introduction to the contaminated room (Figure 5.4).

Six of the 7 calves became diarrhoeic on the first or subsequent day of onset of faecal shedding of Cryptosporidium sp. Calf 4 did not become diarrhoeic, but showed inappetance for 9 days. The most consistent clinical features were depression, anorexia and a severe watery diarrhoea which lasted 2 to 5 days and was terminated either by necropsy (calves 1, 5 and 6) or amelioration of symptoms (calves 2, 3 and 7). Intermittent diarrhoea continued to be observed in calves 3 and 7, which were kept until they were 19 and 24 days of age respectively. Diarrhoeic faeces were yellow and watery and occasionally contained mucus and flecks of blood. Dehydration was only apparent in calves 3 and 7 but after a few days of reduced food intake all the calves had markedly lost condition.

Histology

The histological findings are summarised in Table 5.8. No significant pathological changes or evidence of Cryptosporidium sp infection were seen at any intestinal site in calves 2 and 7.

The parasite could be demonstrated in the mid-gut and terminal ileum of all the calves showing infection at necropsy with organisms being particularly numerous in the mid-gut (Table 5.8). The upper jejunum was infected in only 3 calves (calves 1, 5 and 6) and the large intestine in 2 calves (calves 4 and 5). Cryptosporidium sp were distributed on the surface of the tips and sides of villi and were not seen in the crypts of Lieberkuhn.

The location of Cryptosporidium sp correlated with the presence of histological changes. Typical changes included stunting and

Table 5.8 Histological observations of the intestines of
Cryptosporidium sp infected calves

| <u>Cryptosporidium sp presence and pathological changes in</u> | | | | | | |
|--|----------|---------------|---------|-------------------------------|----------------------------|-----------------|
| Calf number | Duodenum | upper jejunum | mid gut | ileum without Peyer's patches | ileum with Peyer's patches | large intestine |
| 1 | - | C*+++ | C++ | C+ | C*+++ | - |
| 2 | - | - | - | - | - | - |
| 3 | - | + | C+ | C+++ | C+++ | - |
| 4 | - | - | C*+++ | C*+++ | C*+++ | C+ |
| 5 | - | C++ | C*++ | C++ | C+++ | C- |
| 6 | + | C+++ | C*+++ | C++ | C*+ | - |
| 7 | - | - | - | - | - | - |

C; Cryptosporidium sp present on mucosal surface.

C*; heavy infection with Cryptosporidium sp.

-; no significant mucosal changes.

+++; severe mucosal changes including widespread stunting, fusion, epithelial cell cross-bridging, replacement of enterocytes by immature cells and increased cellularity of lamina propria.

++; moderately severe mucosal changes.

+; mild mucosal changes including minor stunting of villi with increased cellularity of the lamina propria.

fusion of adjacent villi, epithelial cell crossbridging, replacement of mature columnar enterocytes with immature cuboidal cells and infiltration of the lamina propria with neutrophils, eosinophils and macrophages (Figure 5.5b). Figure 5.5a shows normal calf ileum for comparison with Figure 5.5b.

Electron microscopy

Examination of tissue from the ileum of calf 4 by scanning electron microscopy (SEM) revealed an extensive infection in which organisms covered the tips and sides of villi (Figures 5.6a and 5.6b). Stunted villi and epithelial cell crossbridging were also apparent (Figure 5.6a).

Examination of the same site by transmission electron microscopy (TEM) showed various developmental stages of Cryptosporidium sp at a pericellular location on the epithelial cells. There was also evidence of denudation of microvilli on epithelial cells in the area of attachment of the organism (Figures 5.7a and 5.7b).

Enzymology

The results for lactase enzyme levels are shown in Table 5.9. As uninfected control calves were not available in this experiment the results are presented as a comparison between those calves with infected intestines at necropsy (calves 1, 3, 4, 5 and 6) and those in which Cryptosporidium sp could not be detected at necropsy (calves 2 and 7). The mean lactase levels of infected animals tended to be lower than those from the recovered animals at each site sampled, but these differences were not significant at $P = 0.05$.

DISCUSSION

Five of 6 experimentally infected calves developed diarrhoea

Table 5.9 Lactase enzyme levels at 3 sites
in the small intestine of calves
infected with Cryptosporidium sp

| calf number | lactase activity, μ moles/min/g wet weight in | | |
|----------------|--|------------|---------------------------------|
| | upper jejunum | mid gut | ileum without Peyers patches |
| 1 | 10.18 | 9.50 | 0.18 |
| 2 | 16.5 | 42.6 | 32.2 |
| 3 | 17.28 | 10.42 | 3.61 |
| 4 | NT | 38.16 | 10.38 |
| 5 | 14.05 | 8.08 | 4.87 |
| 6 | 4.44 | 1.81 | 4.44 |
| 7 | 30.00 | 15.1 | 2.5 |

NT; not tested.

Figure 5.5a Ileum of a normal uninfected calf. There is some artefactual separation of epithelium at the tips of villi. (HE, x 60)

Figure 5.5b Ileum of SPF calf 4 infected with Cryptosporidium sp, killed at 22 days p.i. Note stunting and fusion of villi and cellular infiltration of the lamina propria. (HE, x 150)

125b

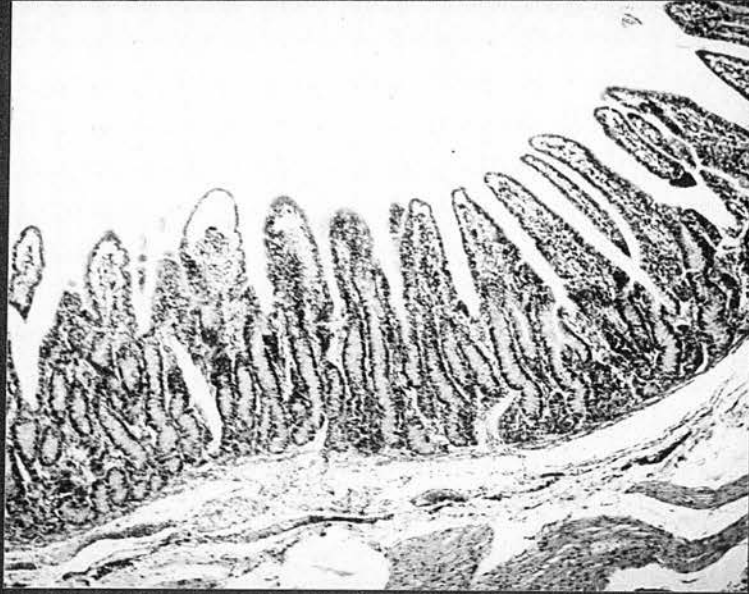


Figure 5.6a Scanning electron micrograph of the ileum of SPF calf 4 infected with Cryptosporidium sp. Villi are stunted and the tips are verrucous. Note the epithelial cell bridges (arrows) between villi. Numerous cryptosporidia can be seen embedded in the surface. (x900)

Figure 5.6b Higher magnification of ileum of SPF calf 4, showing Cryptosporidium sp embedded in the microvillous borders of enterocytes. Note the merozoite (arrowed). (x 6000)

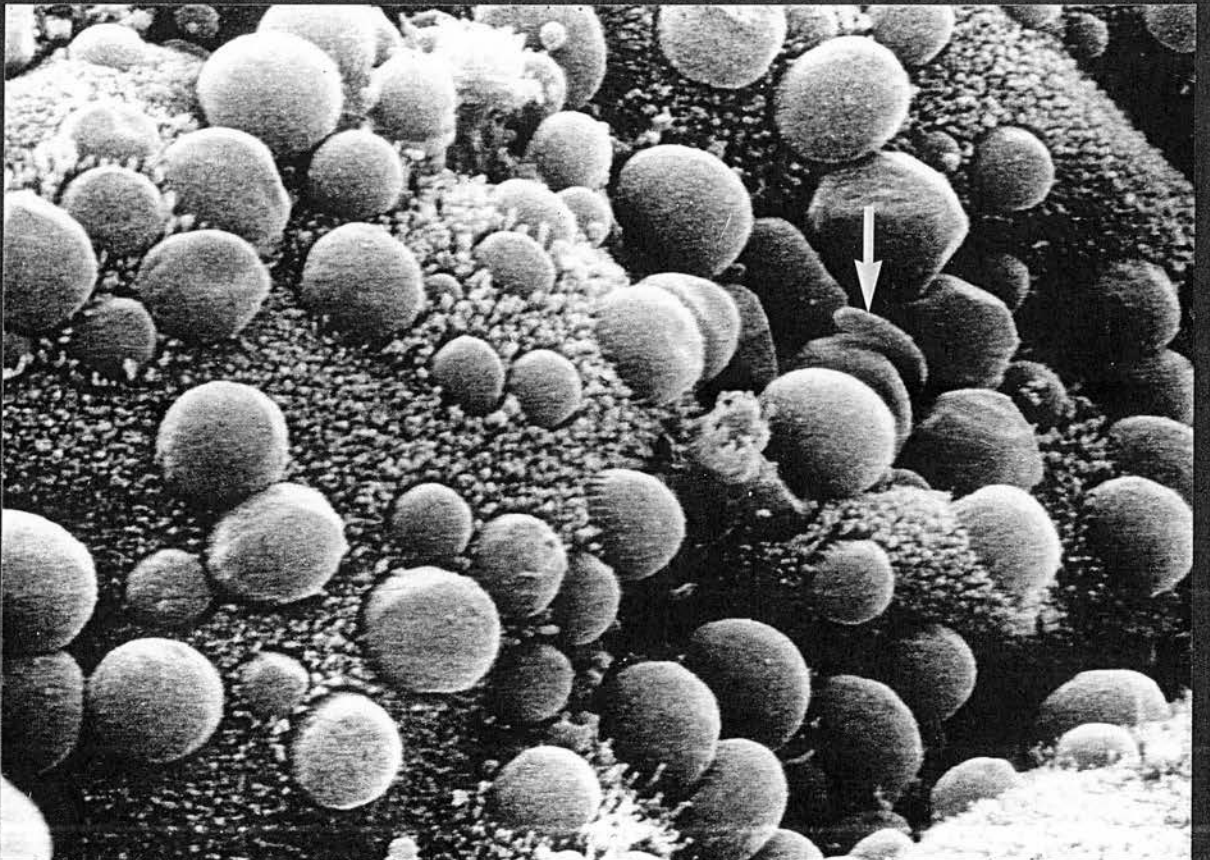
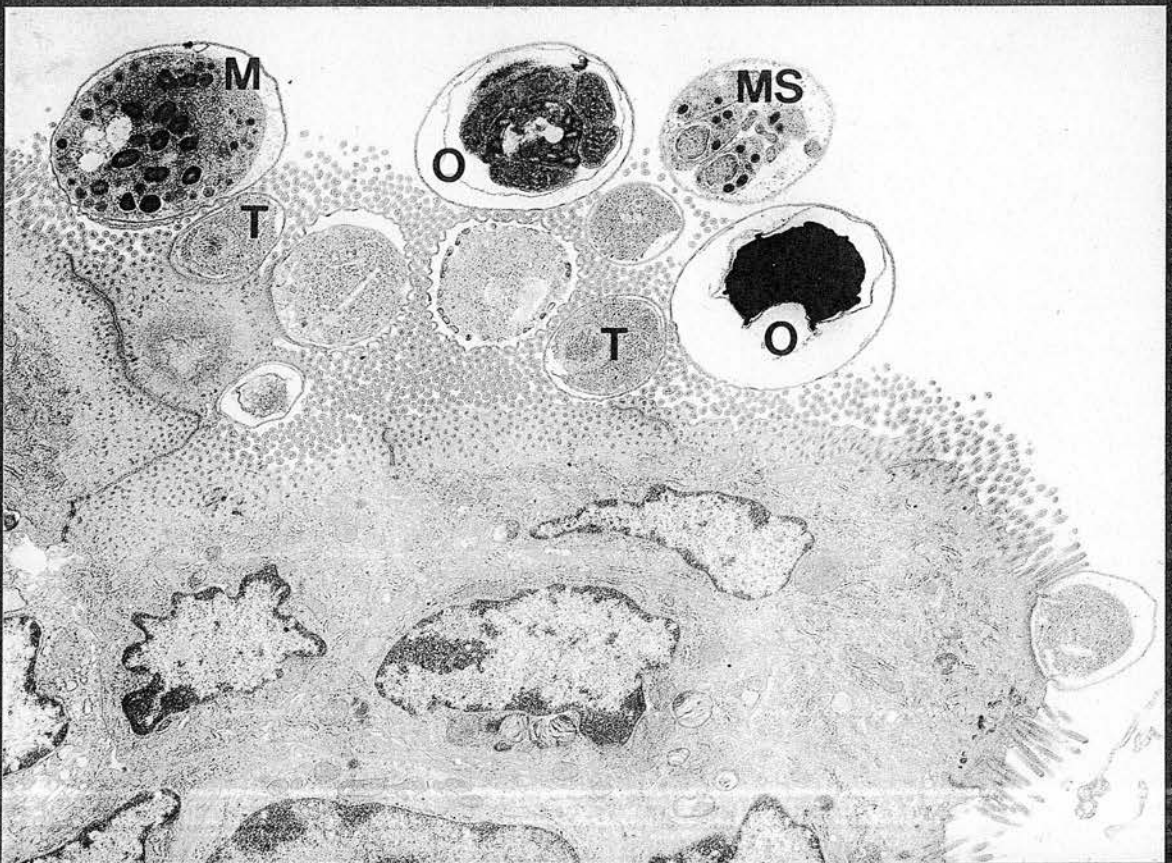
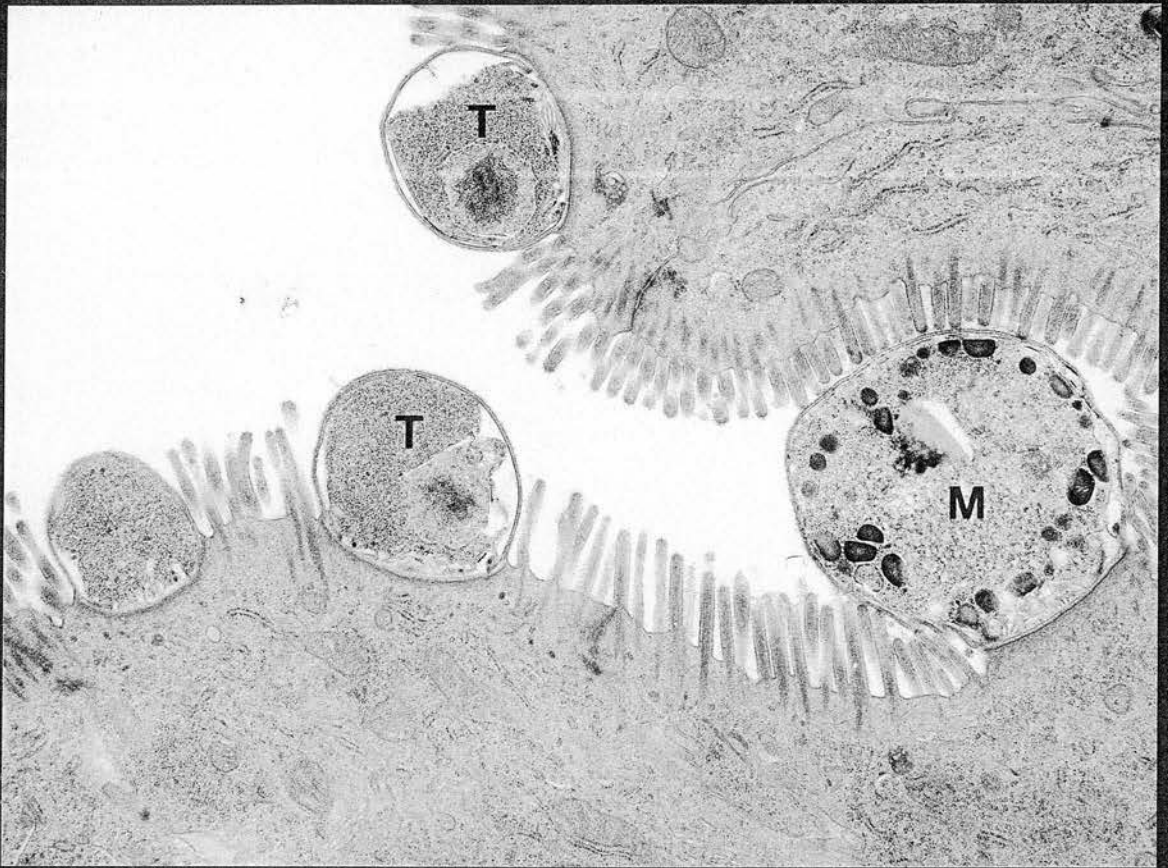


Figure 5.7a Transmission electron micrograph of the ileum of SPF calf 4 infected with Cryptosporidium sp. Trophozoites (T) and a macrogamete (M) are shown attached to the mucosal surface. Microvilli are absent at the sites of attachment. (x12,000)

Figure 5.7b Transmission electron micrograph of the ileum of SPF calf 4 infected with Cryptosporidium sp. Trophozoites (T), macrogamete (M), mature shizont (MS) and oocyst (O) are shown. The free appearance of many of the organisms is an artefact due to the plane of section. (x6,000)



which coincided with faecal shedding of Cryptosporidium sp. The sole calf (calf 4) which did not become diarrhoeic, did not excrete Cryptosporidium sp in the faeces although the organism was present in the intestine at necropsy 12 days p.i. Calf 7 exposed to a Cryptosporidium sp contaminated environment presumably became infected soon after entry to the isolation room as the organism was excreted in the faeces 5 days post entry. This emphasises the infectious nature of this parasite.

Calves were diarrhoeic for 2 to 5 days but did not become severely ill and dehydration was only apparent in two calves (calves 3 and 7). These observations are similar to those reported by Moon and Bemrick (1981) who used conventionally reared colostrum fed calves, but contrast with the severe disease produced in lambs by experimental Cryptosporidium sp infections (Chapter 6, Section A this thesis; Tzipori, Angus, Gray, Campbell and Allan, 1981).

Clinical signs correlated with histological lesions observed in calves, 1, 3, 5 and 6, but inconsistencies were apparent in calves 2 and 4. No histological evidence of infection was seen in calf 2, although Cryptosporidium sp was excreted in its faeces until necropsy. Since diarrhoea was observed in this calf up to 24 hours before necropsy, it seems unlikely that infection had been eliminated; more probably infection was localised at sites not sampled, or was mild. Moon and Bemrick (1981) reported that the intestines of calves killed at 1 to 7 days after cessation of faecal shedding were histologically normal. This could explain the lack of pathological changes observed in calf 7 killed 7 days after oocyst shedding had

stopped. Inappetance was evident for 9 days p.i. in calf 4 but neither diarrhoea nor faecal shedding of Cryptosporidium sp were observed for 12 days p.i. Histological examination of the intestines taken from calf 4 at 12 days p.i. revealed a heavy Cryptosporidium sp infection in the distal small intestine which was associated with marked mucosal changes (Table 5.8). The changes at this site were comparable to those of calves 5 and 6 which were killed whilst diarrhoeic. However, unlike calves 5 and 6, the jejunum of calf 4 was normal, and this might explain the absence of diarrhoea. The reason for the failure to detect faecal oocysts in calf 4 cannot be explained, the animal must have been infected some days before necropsy.

A close association was apparent between Cryptosporidium sp infection of any particular site and the histopathological changes in the mucosa of that site. The most severe changes were in the distal small intestine. Infection extended to the anterior small intestine and on occasions to the colon and caecum, but mucosal changes at these sites tended to be moderate or mild. These observations concur with those of Moon and Bemrick (1981) and suggest that in calves infection may occur initially in the distal small intestine, then ascend the small intestine as the infection progresses. Recycling of the organism by the faecal-oral route may also take place as suggested by Barker and Carbonell (1974).

The TEM and SEM findings are in agreement with previous studies (Pearson and Logan, 1978a; Pohlenz, Bemrick, Moon and Cheville, 1978) and confirm the histopathological changes observed by light microscopy. SEM allowed a more extensive examination of

the mucosal surface as compared with light microscopy and TEM. The different stages in the life cycle of Cryptosporidium sp, its pericellular location and the consequent denudation of microvilli were clearly demonstrated by TEM. TEM studies also confirmed that the bodies present in histological stained sections were Cryptosporidium sp; other, undescribed organisms may have similar appearances by light microscopy.

Both SPF and conventionally-reared calves were shown to be susceptible to infection, although the 5-day-old SPF calves were less severely affected than the two 10-day-old conventional calves. Conventional calves are susceptible to experimental Cryptosporidium sp infections at 21 days of age (Moon and Bemrick, 1981). Thus these limited experiments indicate that the immunoglobulin status and age appear to have no bearing on the development of infection and disease in calves. This is in contrast to experimental studies in SPF lambs (Tzipori, Angus, Gray, Campbell and Allan, 1981), in which a severe Cryptosporidium sp infection was established with clinical illness in newborn lambs, but only subclinical infections were produced in lambs of 30 days of age.

Field surveys have associated Cryptosporidium sp with outbreaks of calf diarrhoea (Pohlenz, Moon, Cheville and Bemrick, 1978; Tzipori, Campbell, Sherwood, Snodgrass and Whitelaw, 1980; Heine and Boch, 1981; Jerret and Snodgrass, 1981). In two of these surveys, one of a single outbreak (Tzipori, ^{Campbell} et al, 1980), 40 to 60% of diarrhoeic calves, but only 0 to 14% of apparently normal calves were excreting Cryptosporidium sp in their faeces.

Calves infected with Cryptosporidium sp have generally been aged between 5 days and 1 month, although there is one report of infection in an 8-month-old animal (Panciera, Thomassen and Garner, 1971). Only one report has implicated Cryptosporidium sp as the sole enteropathogen isolated from an outbreak of calf diarrhoea (Tzipori, Campbell, Sherwood, Snodgrass and Whitelaw, 1980). Most other surveys have found the organism to be involved in mixed infections, either with ETEC, rotavirus or coronavirus (Acres, Laing, Saunders and Radostits, 1975; Morin, Lariviere and Lallier, 1976; Pohlenz, Moon, Cheville and Bemrick, 1978; Nagy, Antal and Lakner, 1980; Snodgrass, Angus, Gray, Keir and Clerihew, 1980; Jerret and Snodgrass, 1981). Whether Cryptosporidium sp shows synergism with other enteropathogens is unknown, but calves during their first 3 weeks of life are particularly likely to develop mixed enteric infections since they are susceptible to disease caused by ETEC, rotavirus and coronavirus.

None of the attempts to treat Cryptosporidium sp infections in calves have been totally successful (Panciera, Thomassen and Garner, 1971; Snodgrass, Angus, Gray, Keir and Clerihew, 1980; Tzipori, Campbell, Sherwood, Snodgrass and Whitelaw, 1980), although partial success was achieved by using sulphadimidine (Snodgrass, Angus, Gray, Keir and Clerihew, 1980). Moon, Woode and Ahrens (1982) have recently attempted chemoprophylaxis of Cryptosporidium sp infections in calves using amprolium, sulphadimidine, dimetridazole, metronidazole, ipronidiazole, quinacrine, monensin and lasalocid but failed to obtain any beneficial effect with any of the drugs used. In this context the mouse model described in Section A of this chapter could

prove useful as a preliminary screening method for evaluating potential drugs, as shown by Tzipori, Campbell and Angus (1982).

CHAPTER 6 SECTION A
EXPERIMENTAL INFECTIONS IN GNOTOBIOTIC AND SPF LAMBS WITH
ETEC, ROTAVIRUS AND CRYPTOSPORIDIUM SP

INTRODUCTION

Gnotobiotic lambs were used to investigate the effects of orally administered ETEC, rotavirus and Cryptosporidium sp both as single and dual infections.

ETEC infections in conventional lambs and calves are very similar as both species of animals are susceptible to clinical infections with K99⁺ ETEC when less than 24 hours of age, but by 48 hours only subclinical infections can be established (Smith and Halls, 1967a). Dual ETEC and rotavirus infections have been studied in gnotobiotic calves (Gouet, Contrepois, Dubourguier, Riou et al, 1978) and colostrum deprived conventional lambs (Wray, Dawson, Afshar and Lucas, 1981) when less than 24 hours old, and prior rotavirus infection in 5 to 8-day-old gnotobiotic calves (Runnels, Moon, Whipp, Matthews and Woode, 1980) was shown to render calves susceptible to clinical infections by ETEC. This latter effect was not examined in lambs, thus experiments were carried out to investigate whether prior rotavirus or Cryptosporidium sp infection would render lambs susceptible to ETEC infections.

Aspects of this work were undertaken jointly with Dr. S. Tzipori at the Moredun Research Institute.

MATERIALS AND METHODS

Derivation of gnotobiotic and SPF lambs

All lambs were hysterectomy-derived, colostrum-deprived as described by Hart, Mackay, McVittie and Mellor (1971). Nineteen lambs were maintained under gnotobiotic conditions in plastic

isolators and 2 were kept under SPF conditions without contact with other animals. All lambs were fed reconstituted evaporated cows milk 3 times daily for the first 3 days and twice daily thereafter.

Inocula

All inocula were administered orally in 2ml volumes.

(a) ETEC

The ovine ETEC strain EC6 (08:K87:K99) was grown for 4 hours in 10ml TSB at 37 °C. The inoculum for lamb 2 (Table 6.1) contained 2.5×10^8 cfu, and that for all other lambs contained 2.0×10^{10} cfu.

(b) Rotavirus

A 20% (v/v in distilled water) faecal filtrate (Millipore filter, 0.45µm average pore diameter) of the sixth passage of lamb rotavirus in gnotobiotic lambs was used (Snodgrass, Smith, Gray and Herring, 1976).

(c) Cryptosporidium sp

Cryptosporidium sp was originally obtained from the faeces of a diarrhoeic calf which were found to be free of ETEC and rotavirus. This organism had been passaged once in suckling SPF rats and twice in SPF lambs (Tzipori, Angus, Gray, Campbell and Allan, 1981). The inoculum comprised a homogenate of gut contents (20% v/v in PBS) prepared from the second SPF lamb in which ETEC and rotavirus were not detected, but in which other unidentified gut flora were present.

Inoculation of lambs

(a) Single infections Lambs up to 8 days of age were inoculated with ETEC (4 lambs), rotavirus (5 lambs) or Cryptosporidium sp (4 lambs) as shown in Table 6.1.

(b) Mixed infections Lambs from 4 to 8 days of age were inoculated with either ETEC and rotavirus (2 gnotobiotic and 2 SPF lambs), rotavirus and Cryptosporidium sp (2 lambs) or ETEC and Cryptosporidium sp (2 lambs) as shown in Table 6.1.

(c) Control lambs Two uninoculated gnotobiotic lambs provided control feed intake and microbiological data. In addition, control data for enzymology was obtained from 5 uninfected lambs which were in use in another experiment, but kept under identical conditions.

Clinical and microbiological monitoring

Lambs were observed twice daily for clinical signs of illness as detailed in Chapter 5, Section B, and the milk consumption of lambs 3 to 6, 11, and 15 to 21 was measured. Faeces samples were collected daily and examined by electron microscopy for enteric viruses, by ELISA for rotavirus, by minca-Isovitalex culture for ETEC and by examination of Giemsa stained faecal smears for Cryptosporidium sp. These techniques have been previously described (Chapter 2).

Necropsy

Intestinal tissue was sampled as described in Chapter 2, except that material was not prepared for transmission or scanning electron microscopy.

Immunofluorescence

The indirect immunofluorescence test was performed as described in Chapter 2 using rabbit antiserum to ETEC strain EC6 or rabbit antiserum against lamb rotavirus (supplied by Dr. D.R. Snodgrass), both diluted 1/20 in PBS. Fluorescein conjugated sheep anti-rabbit immunoglobulin (Wellcome Reagents) was used as indicator.

Table 6.1 Response of gnotobiotic and SPF lambs to oral inoculations with ETEC, lamb rotavirus and Cryptosporidium, singly or in dual infections

| Lamb number | Age at inoculation (days) with | | | Clinical illness ^a | Faecal Shedding of Organisms ^b | Age of lamb at necropsy (days) |
|-----------------|--------------------------------|----|------|-------------------------------|---|--------------------------------|
| | Crypto | RV | ETEC | | | |
| 1 ^c | | | | - | - | 6 to 17 |
| 2 ^d | | | <1 | + | + | <1 |
| 3 | | | 5 | - | + | 8 |
| 4 | | | 5 | - | + | 10 |
| 14 | | | 8 | - | + | 12 |
| 9 | | 1 | | + | + | 4 |
| 10 | | 2 | | + | + | 5 |
| 11 | | 7 | | - | + | 19 |
| 15 | | 8 | | - | + | 10 |
| 16 | | 8 | | - | + | 12 |
| 12 | 1 | | | + | + | 4 |
| 13 | 1 | | | + | + | 6 |
| 17 ^e | 6 | | | + | + | 12 |
| 18 | 6 | | | + | + | 8 |
| 5 | | 4 | 5 | - | + | 8 |
| 6 ^f | | 4 | 5 | - | + | 10 |
| 7 ^f | | 7 | 7 | - | + | 15 |
| 8 ^f | | 7 | 7 | - | + | 16 |
| 19 | 6 | | 8 | + | + | 10 |
| 20 | 6 | | 8 | + | + | 12 |
| 21 | 6 | 8 | | + | + ^g | 10 |
| 22 | 6 | 8 | | + | + ^g | 12 |

a; +, clinical illness.
-, clinically normal.

b; +, faecal shedding of inoculated organisms.
-, no shedding.

c; consisted of 7 uninoculated control lambs.

d; lamb was infected at 6 and killed at 18 hours of age.

e; lamb died as a result of infection.

f; SPF lamb.

g; only Cryptosporidium sp shed in the faeces.

Enzymology

Portions of the duodenum, mid gut and lower ileum from lambs 6, 14 to 16 and 18 to 22 were analysed for lactase, maltase and sucrase by the method described in Chapter 2. The analyses were carried out by M. Gordon, Western General Hospital, Edinburgh.

Enumeration of bacteria

Bacterial counts on mucosal scrapings were performed as described in Chapter 2. Counts from the intestines of ETEC only and ETEC/rotavirus inoculated lambs relate only to the challenge ETEC strain, but counts for any lamb inoculated with Cryptosporidium sp relate to lactose fermenting colonies, presumably E. coli, which were present in the Cryptosporidium sp inoculum.

RESULTS

Clinical and microbiological findings

(a) Control lambs

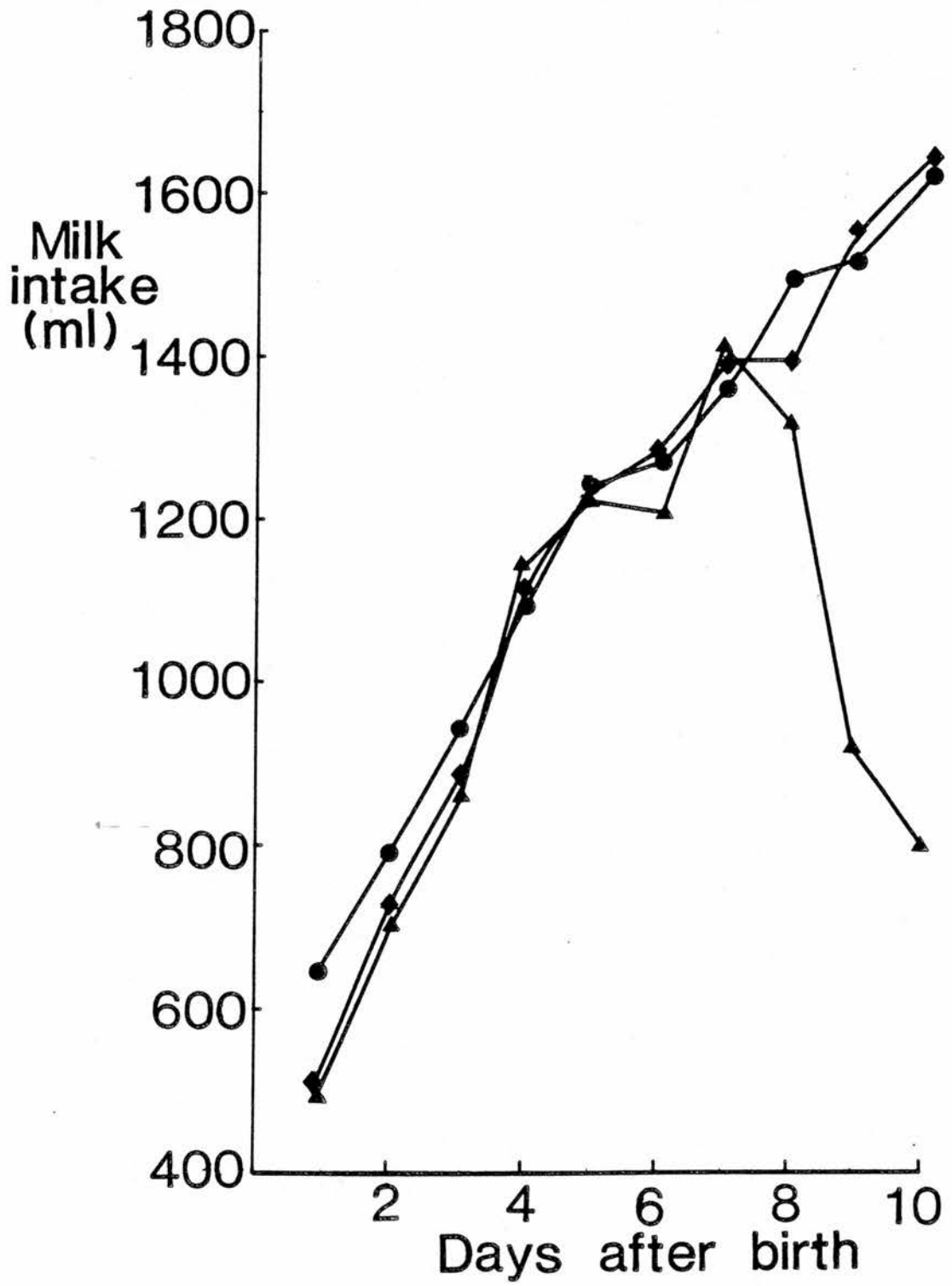
The milk intake of 2 uninoculated lambs is shown in Figure 6.1. Neither diarrhoea nor faecal excretion of enteropathogens were observed in these lambs.

(b) Single agent experiments

(i) ETEC Lamb 2 inoculated 6 hours after birth developed profuse watery diarrhoea within 12 hours p.i. K99⁺ E. coli were present in the faeces of this lamb when it was killed 18 hours after birth. The other 3 lambs (lambs 3, 4 and 14) inoculated with 2×10^{10} cfu at 5 to 8 days of age, remained clinically normal with a normal appetite (Figure 6.1) although they excreted K99⁺ STa⁺ E. coli in their faeces until they were killed.

Figure 6.1 Average daily milk intake of lambs.

- — ● lambs subclinically infected with ETEC and/or lamb rotavirus (data available for lambs, 3, 4, 5, 6, 11, 15 and 16).
- ▲ — ▲ lambs infected with Cryptosporidium sp only, and sometimes with ETEC or rotavirus (data available for lambs, 17, 18, 19, 20 and 21).
- ◆ — ◆ 2 uninoculated control lambs.



(ii) Rotavirus Five lambs (lambs 9, 10, 11, 15 and 16) were inoculated with lamb rotavirus (Table 6.1). Lambs 9 and 10 inoculated at 1 and 2 days of age respectively developed diarrhoea, depression and anorexia with virus excretion continuing until necropsy at 4 to 5 days of age. Lambs 11, 15 and 16 inoculated at 7 to 8 days of age were asymptomatic although rotavirus was detected in their faeces. No decrease in milk intake was observed for lambs 11, 15 and 16 (Figure 6.1).

(iii) Cryptosporidium sp Four lambs (lambs 12, 13, 17 and 18) were inoculated with faecal homogenates containing Cryptosporidium sp (Table 6.1). Lambs 12 and 13 inoculated at 1 day of age, developed profuse watery diarrhoea which soon became intermittent. Lamb 13 became moribund shortly before it was killed at 6 days of age. The milk intake of both lambs was reduced. Lambs 17 and 18 inoculated at 6 days of age became depressed, anorectic (Figure 6.1) and passed soft faeces. Lamb 17 died 2 days p.i. Cryptosporidium sp was detected in the faeces of all 4 lambs at 2 days p.i. and shedding continued until each lamb was necropsied.

(c) Dual infection experiments

(i) ETEC and lamb rotavirus Two gnotobiotic lambs (lambs 5 and 6) and 2 SPF lambs (lambs 7 and 8) were inoculated with ETEC and lamb rotavirus (Table 6.1). All 4 lambs excreted K99⁺ E. coli and rotavirus in their faeces but none became clinically ill, nor was any decrease in milk intake shown by lambs 5 and 6 (Figure 6.1). The milk intake of lambs 7 and 8 were not recorded.

(ii) ETEC and Cryptosporidium sp Two lambs (lambs 19 and 20) inoculated with ETEC and Cryptosporidium sp (Table 6.1) became

depressed by 24 hours p.i. with Cryptosporidium sp. The lambs were diarrhoeic and their milk intake reduced (Figure 6.1). Both K99⁺ E. coli and Cryptosporidium sp were detected in their faeces but subjectively, the clinical effects were no more severe than those produced by Cryptosporidium sp alone.

(iii) Cryptosporidium sp and lamb rotavirus Lambs 21 and 22 were inoculated with Cryptosporidium sp and lamb rotavirus (Table 6.1). Both lambs became diarrhoeic and depressed and their milk consumption fell at 2 days p.i. with Cryptosporidium sp. Cryptosporidium sp were detected in their faeces from 2 days p.i. but rotavirus was not detected in their faeces at any time.

Histology and immunofluorescence

(a) Control lambs

The ileum of an uninoculated control lamb is shown in Figure 6.2. No pathological changes were detected.

(b) Single infection experiments

(i) ETEC Histological examination revealed pathological changes in the intestine of only lamb 2. The duodenum and upper jejunum of this lamb appeared normal and bacterial mucosal adherence was not observed. Bacteria were however seen adhering to the mucosal surface (Figure 6.3) throughout the rest of the small intestine. Pathological changes in the ileum included hyperaemia of mucosal blood vessels, distension of lacteals and infiltrates of neutrophils in the lamina propria. Small deposits of fibrin-like material were occasionally observed at the base of the mucosa and limited cytolysis of lymphocytes was seen in the Peyer patches of the terminal ileum. The spiral colon and caecum were apparently normal.

EC6-specific fluorescence was seen only in lamb 2 where villi from the upper jejunum through to the terminal ileum were coated with bacteria (Figure 6.4).

(ii) Lamb rotavirus Lambs 9 and 10 had lesions associated with rotavirus infections. The villi in the lower small intestine were stunted (Figure 6.5) and the lamina propria was infiltrated by mononuclear cells and eosinophils. Lambs 11 and 16 showed no pathological changes, but the distal small intestine of lamb 15 contained patches of stunted villi which were however clothed by columnar cells. The spiral colon and caecum of all 4 lambs were apparently normal.

Extensive fluorescence specific for rotavirus antigen was found in the epithelial cells of lambs 9 and 10 (Figure 6.6) extending from the lower jejunum to the lower ileum. Occasional single fluorescing cells were seen at sites in the lower small intestine of lambs 11, 15 and 16.

(iii) Cryptosporidium sp The intestines of all 4 lambs showed severe stunting and fusion of villi (Figure 6.7) from the upper jejunum to the lower ileum, with villi clothed with immature cuboidal cells. The lamina propria contained infiltrates of mononuclear cells. The mucosal surfaces of the small and large intestines of all 4 lambs were heavily infected with Cryptosporidium sp; organisms could be readily demonstrated at the tips and sides of villi in the small intestine, but were not evident in the crypt regions.

(c) Dual infection experiments

(i) ETEC and lamb rotavirus No pathological changes were seen at any intestinal site. Immunofluorescence studies for ETEC and

Figure 6.2 Ileum of an uninfected lamb showing normal long slender appearance of villi (x60. HE).

Figure 6.3 Ileum of lamb 2 showing adherent bacteria on the microvillous surface of epithelial cells (x1480, oil immersion, HE).

Figure 6.4 Immunofluorescent staining of the ileum of lamb 2 using antiserum raised against E. coli strain EC6. Note the extensive surface fluorescence (x590. FITC).

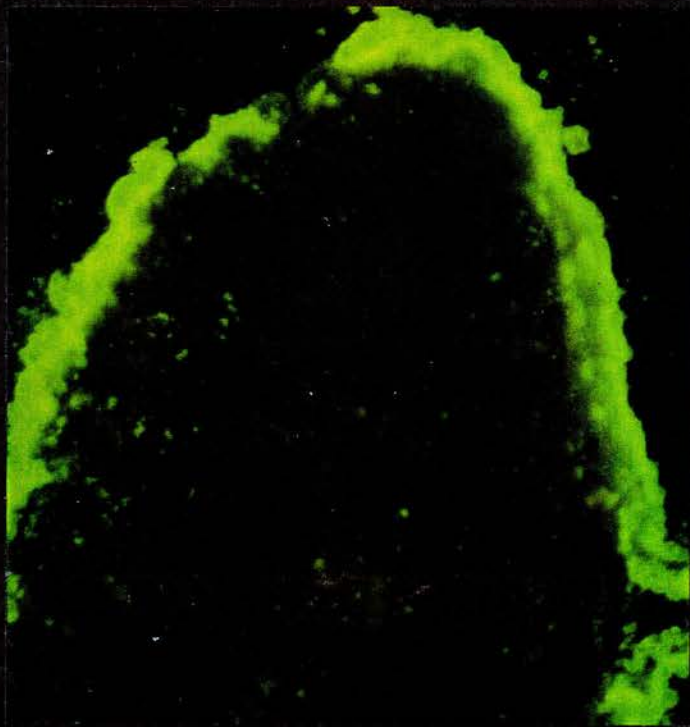
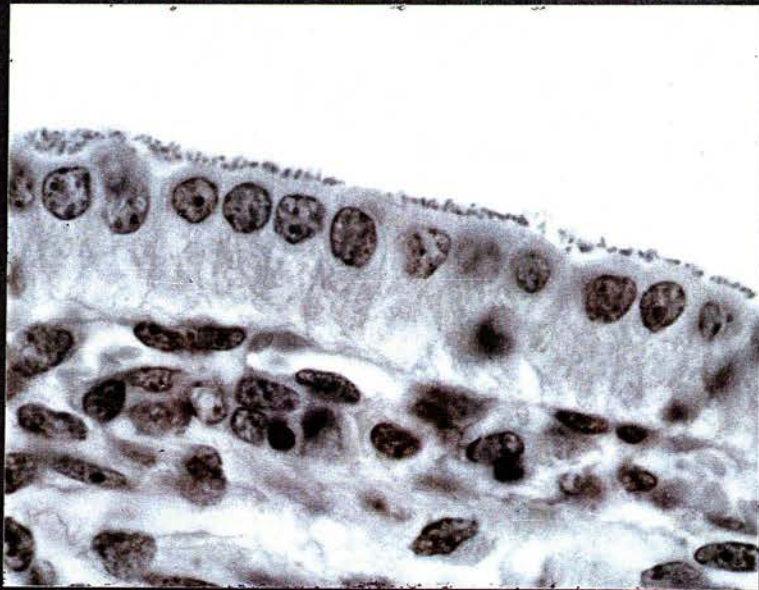
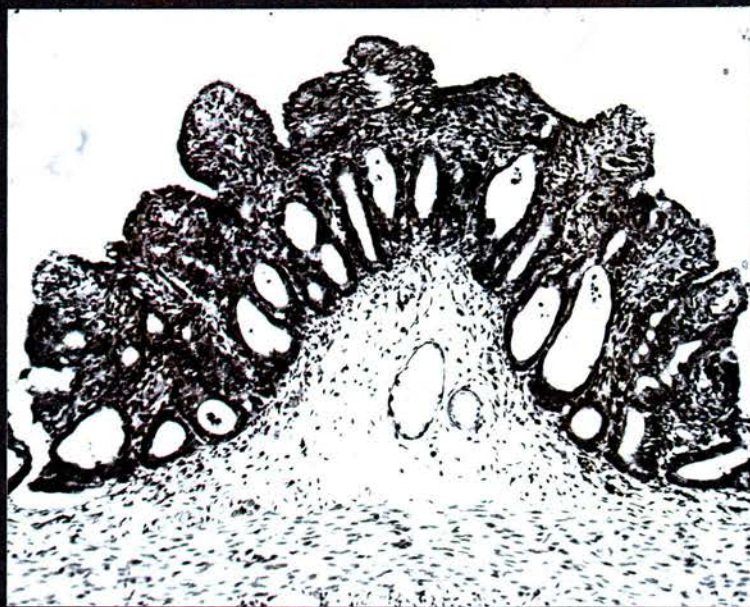
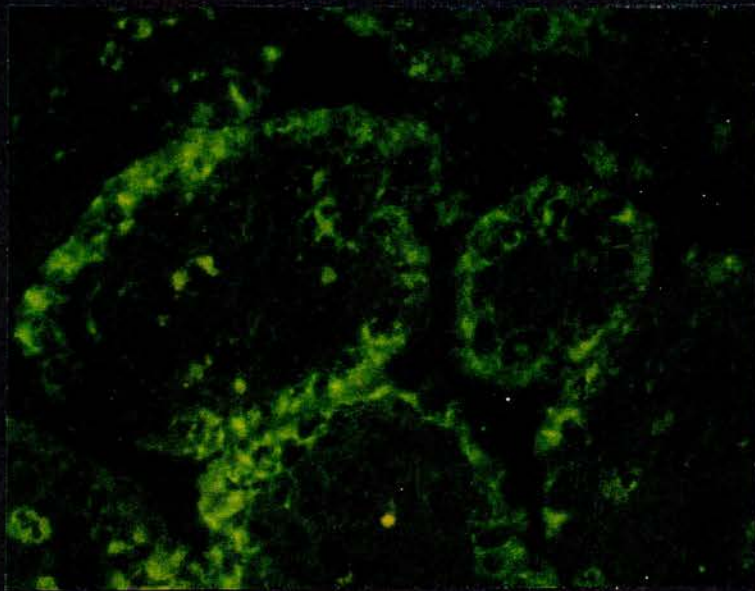
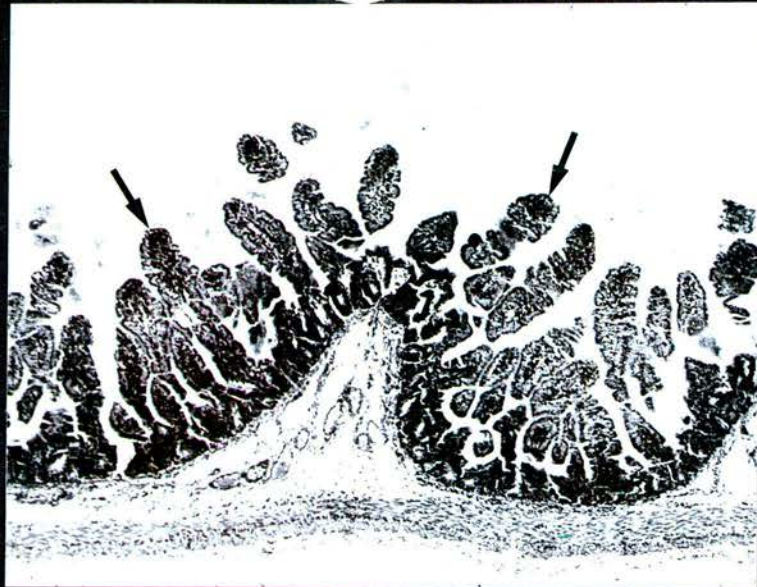


Figure 6.5 Ileum of a lamb infected with lamb rotavirus. Note stunted villi with hypercellular cores (arrowed) (x60, HE).

Figure 6.6 Immunofluorescent staining of lamb ileum using antiserum against lamb rotavirus. Rotavirus antigen present in villous epithelial cells. Note that villi have been cut transversely (x370, FITC).

Figure 6.7 Ileum of a lamb infected with Cryptosporidium sp. Villi are stunted and fused, and crypts are dilated (x150, HE).



rotavirus revealed no specific fluorescence for ETEC strain EC6 although some fluorescence was observed on the surface of the spiral colon and caecum of lambs 7 and 8.

The extent and distribution of rotavirus infected cells detected by fluorescence was similar to that seen in lambs older than 2 days of age infected with rotavirus only (lambs 11, 15 and 16).

(ii) ETEC and Cryptosporidium sp The intestinal lesions in these 2 lambs were similar to those described for lambs inoculated with Cryptosporidium sp alone. Stunting, fusion and epithelial cell bridging of villi were present from the upper jejunum to the terminal ileum. Replacement of enterocytes by immature cells was extensive and infiltration of the crypts by neutrophils from the lamina propria with resultant crypt sepsis was apparent. The small and large intestines were heavily infected with Cryptosporidium sp on the sides and tips of villi only.

The immunofluorescence tests did not reveal any mucosal adherence by the ETEC strain EC6.

(iii) Cryptosporidium sp and rotavirus Lesions found in the intestines of lambs 21 and 22 were similar to those observed for lambs which had been inoculated with Cryptosporidium sp alone or in combination with ETEC. Immunofluorescent examination revealed very few rotavirus infected epithelial cells.

Enumeration of bacteria

Bacterial counts were approximately 10^{10} cfu/0.1g mucosal scraping at small intestinal sites (except the duodenum) taken from lamb 2, but counts from the small intestines of other lambs were

generally lower than 10^8 cfu/0.1g mucosal scraping (Table 6.2).

Enzymology

For statistical and presentation purposes, lambs were grouped into subclinically infected (Group 1), clinically infected (Group 2) and controls (Group 3). Group 1 consisted of lamb 14 (ETEC only), lambs 15 and 16 (rotavirus only) and lamb 6 (ETEC and rotavirus). Group 2 consisted of lamb 18 (Cryptosporidium sp only), lambs 19 and 20 (ETEC and Cryptosporidium sp) and lambs 21 and 22 (Cryptosporidium sp and rotavirus). No material was obtained from the two rotavirus infected lambs 9 and 10. Group 3 consisted of 5 uninoculated age-matched controls.

The group mean and standard error values for sucrase, lactase and maltase activity at different sites along the small intestines are shown in Table 6.3 (individual values are detailed in Appendix 4.). There were no significant differences between or within groups at any site for either maltase or sucrase activity. However, with lactase, significant differences in activity were recorded between groups 1 and 2, and 2 and 3 ($P < 0.05$) in both the duodenum and mid gut.

DISCUSSION

These experiments show that gnotobiotic lambs were susceptible to clinical infection by single infections of rotavirus and ETEC when less than 3 days of age, but only subclinical infections were established with these agents in lambs greater than 4 days of age. A calf Cryptosporidium sp isolate produced disease in all lambs exposed at 1 or 6 days of age.

Table 6.2 Bacterial counts from mucosal scrapings taken at various sites in the small and large intestines of gnotobiotic lambs inoculated with ETEC, lamb rotavirus and Cryptosporidium sp

| log ₁₀ colony forming units/0.1g mucosal scraping at sites taken from | | | | | | | |
|---|----------------|----------|---------------|---------|-------------------------------|----------------------------|------------------------------|
| Infection group | Lamb number | duodenum | upper jejunum | mid gut | ileum without Peyer's patches | ileum with Peyer's patches | large intestine ^a |
| ETEC only | 2 | 7.40 | 9.78 | 9.88 | 10.10 | 10.30 | 10.24 |
| | 3 | 6.32 | ND | 6.70 | ND | 8.48 | 9.40 |
| | 4 | 5.74 | ND | 6.18 | ND | 7.40 | 8.50 |
| | 14 | 5.40 | 6.00 | 6.70 | 8.48 | 8.81 | 8.68 |
| <u>Cryptosporidium</u> sp only ^b | 18 | 3.70 | 7.04 | 4.95 | 6.30 | 7.18 | 7.57 |
| ETEC and lamb rotavirus | 5 | 6.40 | ND | 7.30 | ND | 8.48 | 9.25 |
| | 6 | 3.48 | ND | 6.30 | ND | 7.40 | 9.00 |
| | 7 ^c | ND | 6.90 | 7.30 | 8.70 | 7.70 | 8.90 |
| | 8 ^c | 2.18 | 2.74 | 2.82 | 6.00 | 6.60 | 8.93 |
| ETEC ^d and <u>Cryptosporidium</u> sp | 19 | 5.08 | 6.90 | 6.87 | 6.90 | 5.18 | 8.24 |
| | 20 | 6.18 | 5.09 | 7.54 | 9.00 | 8.84 | 8.15 |
| <u>Cryptosporidium</u> ^b sp and lamb rotavirus | 21 | 5.70 | 7.18 | 7.15 | 8.00 | 7.60 | 7.90 |
| | 22 | 3.70 | 6.00 | 3.70 | 6.48 | 4.30 | 9.00 |
| a; average count found in the spiral colon and caecum | | | | | | | |
| b; counts for lactose fermenting bacteria (presumably <u>E. coli</u>) from the <u>Cryptosporidium</u> sp inoculum | | | | | | | |
| c; SPF lambs | | | | | | | |
| d; counts for ETEC and lactose fermenting bacteria (presumably <u>E. coli</u>) from the <u>Cryptosporidium</u> sp inoculum | | | | | | | |
| ND; not done | | | | | | | |

Table 6.3 Sucrase, lactase and maltase activity (μ moles/min/g wet weight)
in the small intestines of infected and uninfected control
lambs

| Group ^a | enzyme activity in μ moles/min/g wet weight \pm standard error in | | | | | | | | |
|---|--|------------------------------------|-----------------------|-----------------------|------------------------------------|-----------------------|-----------------------|-----------------------|-----------------------|
| | duodenum | | | mid gut | | | lower ileum | | |
| | S | L | M | S | L | M | S | L | M |
| 1 (subclinically infected, n = 4) | 0.85 \pm 0.36 | 6.69 \pm 1.61 | 0.26 \pm 0.20 | 0.17 \pm 0.06 | 8.00 \pm 2.03 | 0.20 \pm 0.11 | 0.22 \pm 0.18 | 0.93 \pm 0.42 | 0.38 \pm 0.16 |
| 2 (clinically infected, n = 5) | 0.43 \pm 3.2 | 2.11 ^b \pm 0.60 | 0.52 \pm 0.18 | 0.17 \pm 0.17 | 1.15 ^c \pm 0.28 | 0.36 \pm 0.13 | 0.19 \pm 0.04 | 0.28 \pm 0.14 | 0.23 \pm 0.09 |
| 3 (control, n = 5) | 0.55 \pm 0.34 | 4.65 \pm 0.49 | 0.37 \pm 0.08 | 1.28 \pm 0.66 | 4.33 \pm 0.79 | 0.65 \pm 0.14 | 0.87 \pm 0.87 | 1.85 \pm 1.52 | 0.39 \pm 0.19 |

a; information on grouping of lambs is given in the results section for enzymology

b; $P < 0.05$ for lactase with respect to Group 1 and Group 3

c; $P < 0.05$ with respect to Group 1 and $P < 0.01$ with respect to Group 3

S; sucrase

L: lactase

M; maltase

Only lamb 2 exposed to ETEC exhibited severe enterotoxigenic colibacillosis as described by Sojka, Morris and Wray (1979) and Wray, Dawson, Afshar and Lucas (1981). High bacterial counts in the intestine were obtained from this animal and histological and immunofluorescence studies confirmed the adherence of ETEC strain EC6 to the distal small intestine. Lambs inoculated with a higher dose of ETEC at 5 or 8 days of age (lambs 3, 4 and 14) did not succumb to clinical infection although ETEC were excreted in the faeces. Such an age-related susceptibility has been observed before with experimental enterotoxigenic colibacillosis in calves and lambs (Smith and Halls, 1967a; Tzipori, Makin, Smith and Krautil, 1981).

Lambs inoculated with lamb rotavirus at 1 to 2 days of age became diarrhoeic and showed histological lesions and specific immunofluorescence in the distal small intestine as described previously by Snodgrass, Angus and Gray (1977). However only subclinical infections were produced when the same inoculum was used to infect 7 to 8-day-old lambs. These results are at variance with the clinical disease that has been produced in calves inoculated with rotavirus at 4 to 7 days of age (Runnels, Moon, Whipp, Matthews and Woode, 1980; Tzipori, Makin, Smith and Krautil, 1981; Snodgrass, Smith and Krautil, 1982) and up to 35 days of age (Woode and Crouch, 1978). These results for lamb rotavirus infections should be confirmed using a number of other lamb rotavirus isolates.

All lambs infected with calf Cryptosporidium sp, after passage in SPF rats and SPF lambs, developed severe clinical infections and lesions of the small and large intestines irrespective of age (1 or 6 days) at inoculation. The histological lesions produced were

similar to those described previously in SPF and conventionally reared lambs (Tzipori, Angus, Campbell and Clerihew, 1981; Angus, Tzipori and Gray, 1982). The same calf Cryptosporidium sp used in this present study had previously produced severe diarrhoea in newborn SPF lambs (Tzipori, Angus, Gray, Campbell and Allan, 1981). Lambs of 5 to 20 days of age showed less severe clinical disease, and at 30 days of age subclinical infections only were established.

Bacteriological examination of one lamb infected with Cryptosporidium sp (lamb 18) revealed that the bacteria present in the inoculum had not produced significant colonisation of the gut. It must be stressed that no enteropathogens, in particular rotavirus, coronavirus or ETEC were detected in the Cryptosporidium sp inoculum. The inoculum had been 'biologically filtered' in SPF rats and lambs before use in these experiments to reduce the risk of contamination with other enteropathogens. The Cryptosporidium sp inoculum was not titrated in suckling mice as recommended in Chapter 5, Section A, but it did remain infective for suckling mice over the 2 month experimental period although there was almost certainly a decrease in its infective titre. Fortunately, the inoculum was stored in PBS at 4 °C, one of the better storage methods found in Chapter 5, Section A.

Neither gnotobiotic lambs inoculated with ETEC at 4 days of age followed by rotavirus 1 day later, nor SPF lambs simultaneously inoculated with both organisms at 7 days of age developed clinical disease although both enteropathogens were excreted in faeces. Mixed ETEC and rotavirus infections in colostrum deprived lambs under 24 hours of age were found by Wray, Dawson, Afshar and Lucas

(1981) to be more severe than single infections; comparable results were obtained for gnotobiotic calves infected under 24 hours of age (Dubourguier, Gouet, Mandard, Contrepolis and Bachelerie, 1978; Gouet, Contrepolis, Dubourguier, Riou, et al, 1978). Mixed ETEC and rotavirus infections have been studied in 5 to 8-day-old gnotobiotic calves (Runnels, Moon, Whipp, Matthews and Woode, 1980) and 7-day-old conventionally reared calves (Snodgrass, Smith and Krautil, 1982). It was suggested that the age of calves and sequence of inoculation of ETEC and rotavirus were important factors to consider in mixed enteric infections involving these two organisms. In this present study, the sequence used for ETEC and rotavirus was that considered optimal for calves, namely simultaneously or 1 day apart, but the delay of necropsy to 3 to 9 days post inoculation with ETEC may have allowed adherence of ETEC, if it occurred, to subside. Bacterial counts from lambs subclinically infected with ETEC were similar to lambs subclinically infected with ETEC and rotavirus. Thus enhanced adherence was not apparent in this instance. Bacterial counts cannot be compared with those found in lamb 2 as this lamb was killed at 12 hours post inoculation with ETEC. The dual ETEC and Cryptosporidium sp infected lambs were killed at 2 and 4 days post inoculation with ETEC, but there was no adherence of ETEC to the intestines of these lambs, and subjectively the clinical illness observed was similar to that of Cryptosporidium sp only infected lambs.

The enzyme studies suggest that lactase levels were good indicators of intestinal damage which may have occurred during enteric infections. All Cryptosporidium sp infected lambs had significantly decreased lactase levels in the small intestine. This

has also been shown to occur as a consequence of clinical infections induced by rotavirus in gnotobiotic lambs (Ferguson, Paul and Snodgrass, 1981), but this was not measured in the present study. There were no significant differences between the lactase levels of subclinical ETEC only and ETEC/rotavirus infected lambs and between Cryptosporidium sp and ETEC/Cryptosporidium sp infected lambs. Thus lactase levels were not influenced by the dual nature of the infections.

In conclusion, lambs could be subclinically infected with ETEC only, rotavirus only or ETEC and rotavirus at greater than 2 days of age. There was no evidence to suggest that either rotavirus or Cryptosporidium sp allowed an increase in mucosal adherence by ETEC in lambs greater than 2 days of age. The clinical responses observed in these gnotobiotic lambs were different to the responses reported for similar infections in gnotobiotic calves (Runnels, Moon, Whipp, Matthews and Woode, 1980), although these differences may relate to experimental design and strains of enteropathogens used.

CHAPTER 6 SECTION BEXPERIMENTAL INFECTIONS IN CONVENTIONAL CALVES WITHETEC AND CRYPTOSPORIDIUM SPINTRODUCTION

Multiple enteric infections of calves have been observed in field surveys (Chapter 3), but studies to identify the interaction of these agents under experimental conditions have been limited to ETEC and rotavirus infections (Gouet, Contrepois, Dubourguier, Riou et al 1978; Runnels, Moon, Whipp, Matthews and Woode, 1980; Tzipori, Makin, Smith and Krautil, 1981; Snodgrass, Smith and Krautil, 1982).

The following experiment was undertaken in an attempt to investigate dual infections of calves with ETEC and Cryptosporidium sp. It was not possible to undertake the experiment as originally planned, the prime factor responsible being the demonstration of natural rotavirus infection in animals purchased for experiment. These animals had to be discarded thus disrupting the experiment in two ways. Firstly, it necessitated a change in the Cryptosporidium sp inoculum employed as the experiment had to be extended beyond the original predicted period, and secondly, it was not possible to complete the animal infections as had been intended, thus certain groups do not contain sufficient animals. These two factors combine to detract considerably from the experiment; the results are presented in the knowledge of these deficiencies and merely give an indication of the results and problems which may be encountered in undertaking experiments of this type in conventional animals.

MATERIALS AND METHODS

Animals and feeding

Jersey or Ayrshire bull calves from 2 farms were brought to the Moredun Research Institute within 36 hours of birth after they had sucked their dams. Calves were housed separately in isolation rooms and attendants wore protective clothing at all times when feeding or handling. Calves were fed twice daily with evaporated cows milk reconstituted with distilled water in a 3:7 ratio. The feeding regime was; 2.0 l per day up to 7 days of age, 2.5 l per day from 8 to 10-days-old, 3.0 l per day from 11 to 14-days-old and 3.5 l thereafter.

Inocula and inoculation of calves

(a) E. coli

ETEC strain B44 grown in TSB overnight was inoculated orally in 10ml doses. The average dose contained 1.83×10^{10} cfu (range 0.7 to 3×10^{10} cfu).

(b) Cryptosporidium sp

Two calves (E33 and E34) were inoculated orally with 20ml of calf faecal homogenate (20% w/v in 5% BSA) containing Cryptosporidium sp. These 2 calves have been described previously in Chapter 5, Section B as calves 5 and 6 respectively. ETEC, rotavirus, coronavirus and Salmonella spp were not detected in this inoculum using the screening methods described in Chapter 3. Faeces from calf E33 were homogenised 1:1 in 5% BSA and stored for 2 weeks. This inoculum was used to inoculate calves E38 and E39 in 20ml volumes, and was similarly found not to contain any other detectable enteropathogens. Calves F682 and F684 were inoculated orally with 3ml of faecal suspension

(50% w/v in PBS) from a Cryptosporidium sp infected gnotobiotic lamb. This organism was originally obtained from a calf and had been passaged 12 times in inbred Porton mice before inoculation of the lamb (Chapter 5, Section A).

(c) Controls

Two uninoculated calves were kept in separate isolation rooms for the duration of the experiment. One further calf, F609 (described in Chapter 4, Section B) was included for comparison of the numbers of intestinal bacteria. This animal demonstrated severe enterotoxigenic colibacillosis after inoculation of ETEC strain B44 at under 18 hours of age.

(d) Inoculation of calves

The calves used and their age at inoculation are listed in Table 6.4. Calves given one agent were inoculated at 9 days of age and those given both agents were exposed to Cryptosporidium sp at 9 days and to ETEC at 11 days of age.

Clinical observations

The animals were assessed clinically twice daily for the whole experimental period. Faecal consistency was recorded, and in some cases the percentage faecal dry matter content was measured by drying faeces at 100 °C. The daily milk intake was recorded.

Microbiological monitoring

Faecal samples taken daily throughout the experiment were screened for K99⁺ E. coli, Cryptosporidium sp and enteric viruses. Calves which excreted any of the above enteropathogens prior to inoculation or as a contaminant post inoculation (p.i.) were excluded from the experiment. Eight calves were rejected from the

Table 6.4 Age at inoculation and necropsy of conventional calves inoculated with ETEC and *Cryptosporidium* sp

| Breed | Calf No | Age at inoculation (days) with | | Organism shedding | Age at post-mortem (days) | Mancini IgG (mg/ml) |
|-------|---------|--------------------------------|------|-------------------|---------------------------|---------------------|
| | | <i>Cryptosporidium</i> sp | ETEC | | | |
| F | F609 | | 1 | + | 2 | NT |
| A | F695 | | | - | 7 | NT |
| A | F697 | | | - | 10 | 19.1 |
| A | F692 | | 9 | + | 10 | 12.0 |
| A | F693 | | 9 | + | 10 | 66.1 |
| J | E236 | | 9 | + | 13 | 63.5 |
| J | E237 | | 9 | + | 11 | NT |
| A | F682 | 9 | | + | 18 | 38.2 |
| A | F684 | 9 | | + | 18 | 8.9 |
| J | E33 | 9 | | + | 15 | 86.1 |
| J | E34 | 9 | | + | 15 | 43.7 |
| J | E38 | 9 | 11 | + | 13 | 0 |
| J | E39 | 9 | 11 | + ^a | 16 | 27.5 |

A; Ayrshire +; challenge organisms detected in faeces

F; Friesian -; no K99⁺ ETEC, *Cryptosporidium* sp or enteric viruses in faeces

J; Jersey

NT; not tested a; only ETEC detected in faeces

experiment between 2 and 9 days of age; 5 because they developed rotavirus infection, 2 because they had severe diarrhoea which was not associated with any detectable enteropathogens, and one because it excreted unidentified small round viruses.

Necropsy, bacterial counts, immunofluorescence and enzymology

The methods used were described in Chapter 2.

For the indirect-immunofluorescence test (IF) rabbit antisera to E. coli strain B44 or lamb rotavirus diluted 1/20 in PBS were used on duplicate fixed cryostat sections. This latter test was used to confirm the absence of rotavirus in experimental animals.

Lactose tolerance test

This test was carried out on calves F682, F684, F692 and F693 24 hours before inoculation, 1 day p.i. with E. coli strain B44 and 5 days p.i. with Cryptosporidium sp.

Calves were fasted overnight, weighed and orally dosed with 1.5 l of a lactose solution in distilled water containing 2.5g of lactose/kg body weight. The calves were bled at 0, 10, 20, 30, 45, 60, 75, 90 and 120 minutes post-dosing and heparinised plasma was separated and stored at -20 °C until assayed for glucose. The plasma glucose results were converted to whole blood values by a correction factor calculated from the packed cell volume.

Plasma for glucose analysis was deproteinised with 10% trichloroacetic acid (TCA) at a ratio of 1:4. The precipitate was removed by centrifugation for 15 minutes at 1000g at 4 °C. The supernates were assayed for glucose by Dr. C. Hodgson, Moredun Research Institute, by the line method described by Trinder (1969).

Serology

Sera taken from all calves at 24 to 48 hours after birth were tested for K99 antibodies by the sandwich ELISA (Chapter 4, Section A) and for total IgG content by the Mancini technique (Chapter 2).

RESULTS

Microbiology and clinical observations

(a) Uninoculated control calves

Calves F697 and F659 fed well during the experimental period and did not become diarrhoeic. The minimum faecal dry matter content of calf F697 was 26.5%. Neither calf excreted any detectable enteropathogen. They were necropsied at 7 (F697) and 9 (F659) days of age.

(b) Calves inoculated with ETEC only

All 4 calves (E236, E237, F692 and F693) remained alert and clinically normal throughout the experiment (Figure 6.8). The dry matter content of faeces from calves F692 and F693 did not fall below 23.0%. All calves excreted the challenge strain B44 from 24 hours p.i. until necropsy (calves F692 and F693 at 36 hours p.i., calf E237 at 60 hours p.i. and E236 at 4.5 days p.i.). The organisms isolated were K99⁺ and STa⁺.

Bacterial counts at 7 intestinal sites in the 4 calves are shown in Table 6.5. The results show the total coliform population present and indicate that coliform levels in the 9-day-old inoculated calves were much lower than those of calf F609 which had developed severe enterotoxigenic colibacillosis at 18 hours old.

(c) Calves inoculated with *Cryptosporidium* sp only

Calves E33 and E34 became anorectic and had severe watery diarrhoea at 3 days p.i. which lasted for 3 days (Figure 6.8). At

Table 6.5 Bacterial counts at 7 intestinal sites of calves inoculated with






ETEC only or Cryptosporidium sp and ETEC

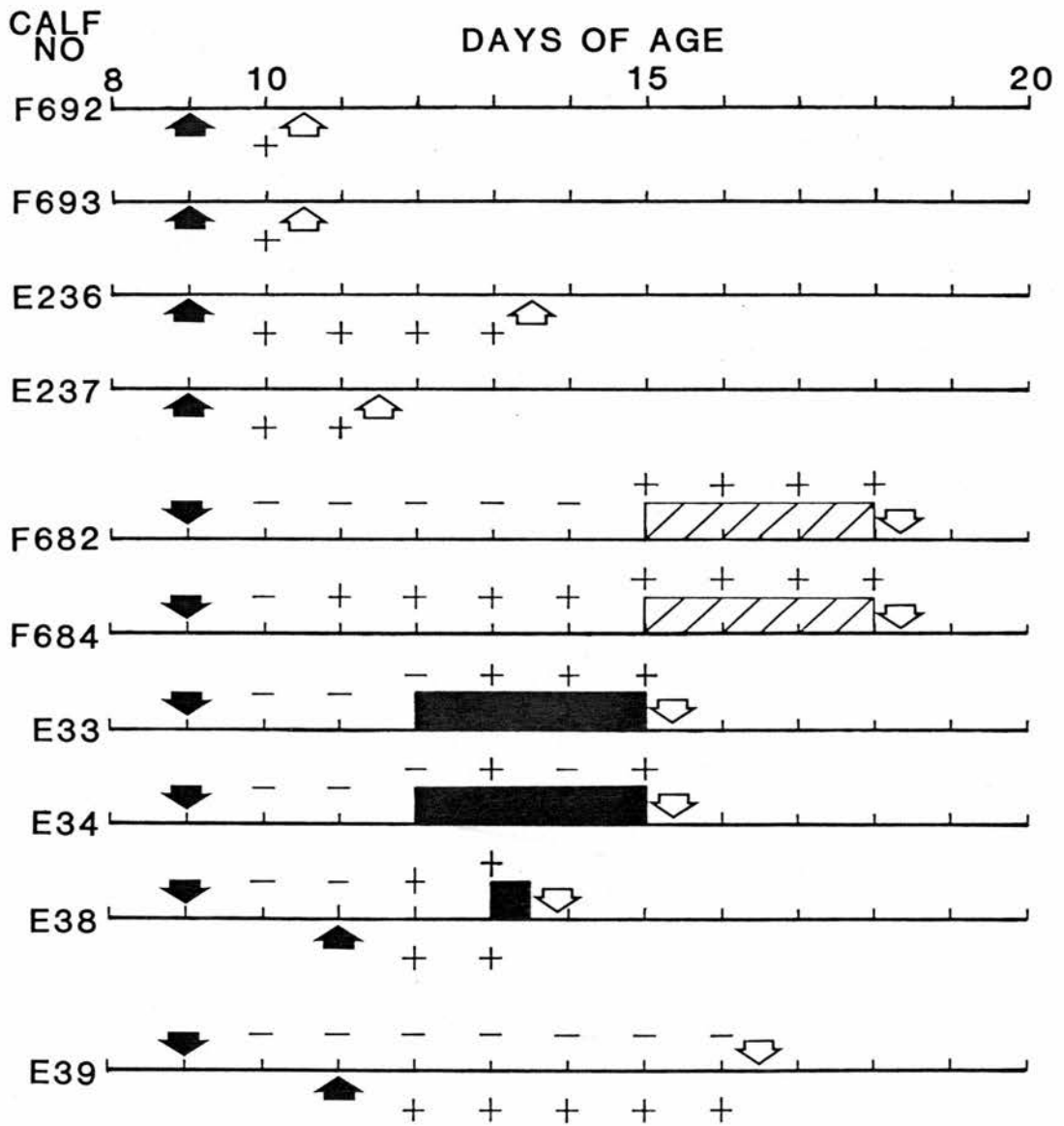
| Intestinal Site | \log_{10} cfu/0.1g of mucosal scraping for all lactose fermenting coliforms present in calves infected with | | | | | | | | | |
|---------------------------------|--|------|------|------|-------|------------------------------------|------|--|--|--|
| | ETEC only | | | | | ETEC and <u>Cryptosporidium</u> sp | | | | |
| | F692 | F693 | E236 | E237 | F609 | E38 | E39 | | | |
| duodenum | NC | NC | NC | NC | 7.40 | NC | NC | | | |
| upper jejunum | NC | NC | NC | NC | 9.00 | NC | NC | | | |
| mid-gut | NC | NC | NC | NC | 10.40 | 4.70 | NC | | | |
| ileum without Peyers patches | NC | 4.81 | NC | 2.18 | 10.18 | 4.70 | 5.30 | | | |
| ileum with Peyers patches | 4.70 | 4.70 | 6.30 | 2.00 | 10.00 | 7.00 | 6.78 | | | |
| Large ^a intestine | 6.41 | 7.80 | 6.63 | 6.00 | 9.85 | 7.20 | 6.44 | | | |

NC; no count at a dilution of 1/100

a; average count of spiral colon and caecum

Figure 6.8 The excretion of enteropathogens and clinical course of infection in calves inoculated with ETEC only, Cryptosporidium sp only or ETEC and Cryptosporidium sp

| | |
|---|--|
|  | Inoculation with <u>Cryptosporidium</u> sp. |
|  | Inoculation with ETEC. |
| $\frac{+}{+}$ | <u>Cryptosporidium</u> sp detected in the faeces ETEC " " " " |
| $\frac{-}{-}$ | <u>Cryptosporidium</u> sp not detected in the faeces ETEC " " " " |
|  | watery diarrhoea. |
|  | mild diarrhoea. |
|  | calf killed. |



necropsy (6 days p.i.) both calves were dehydrated (sunken eyes and tight skin) weak and emaciated. The faecal dry matter content of these calves was not measured.

Calf F682 and F684 remained clinically normal although soft yellow faeces with a dry matter content of 21.5 to 25.0% were passed for 3 days commencing at 6 days p.i.

Excretion of Cryptosporidium sp in the faeces of calves E33, E34, F682 and F684 is shown in Figure 6.8. The organism was first detected in the faeces of calves E33 and E34 at 4 days p.i., in calf F682 at 6 days p.i. and in calf F684 at 2 days p.i.

(d) Calves inoculated with ETEC and Cryptosporidium sp

Calf E39 remained clinically normal except for the excretion of soft yellow faeces for 2 days starting at 2 days p.i. with Cryptosporidium sp. Calf E38 became anorectic, dehydrated and had profuse watery diarrhoea starting at 4 days p.i. (Figure 6.8). Both calves excreted the challenge strain B44 from 24 hours p.i. until necropsy at 60 hours p.i. with ETEC for calf E38 and 5 days p.i. with ETEC for calf E39. Only calf E38 excreted Cryptosporidium sp from 3 days p.i. (Figure 6.8).

Coliform levels in the intestines of both calves at necropsy were much lower than those of calf F609, and were similar to the levels observed in calves inoculated with strain B44 only (Table 6.5).

Histology and IF

(a) Uninoculated control calves

No significant pathological changes were observed at any intestinal site in calves F697 and F659. No evidence of adherent ETEC B44 or of rotavirus infection was found by IF staining.

(b) Calves inoculated with ETEC only

Pathological changes were observed only in the terminal ileum of calves E237, F692 and F693. These changes included swollen and oedematous villi or in some cases almost total villous atrophy with stunting and fusion. A neutrophil and eosinophil infiltrate was observed in the lamina propria and areas of epithelium were cuboidal and necrotic with some exfoliation.

Adherent bacteria were not seen in the intestines of any calf either by histology or IF, nor was there any evidence of rotavirus infection by IF.

(c) Calves inoculated with *Cryptosporidium* sp only

The most severe pathological changes were observed in the small intestine from the upper jejunum to ileum of calves E33 and E34 which were killed at 6 days p.i. The infection with *Cryptosporidium* sp was widespread, the organisms being found on the tips and sides of villi in affected areas. Typical pathological changes included villous stunting and fusion, epithelial-cell cross-bridging, villi clad in cuboidal cells and infiltrates of neutrophils and macrophages in the lamina propria. A small number of cryptosporidia were observed in the caecum of calf E33 and adherent bacteria were seen in the caecum of calf E34.

Pathological changes and infection of calves F682 and F684 were confined to the mid-gut and ileum. Limited villous atrophy and fusion were observed in these areas, with infiltrates of mononuclear cells and neutrophils in the lamina propria. There was no evidence of rotavirus infection in any calf by IF.

(d) Calves inoculated with ETEC and Cryptosporidium sp

Pathological changes in calf E39 appeared only in the ileum where focal necrosis and erosion of villous epithelium was evident in mucosa overlying Peyer's patches. There was no apparent Cryptosporidium sp infection at any site in this calf.

Pathological changes (similar to those described for calves E33 and E34) and evidence of Cryptosporidium sp infection were apparent in the ileum of calf E38. Adherent bacteria were not observed in either calf by histology or IF, and there was no evidence of rotavirus infection by IF examination.

Enzymology

The lactase levels at three sites in the small intestines of all calves except F659 are shown in Table 6.6.

Lactose tolerance test

The results given in Table 6.7 are presented as percentages of the mean starting blood glucose level. The small number of calves examined in each group and the degree of variation within a group prohibited statistical analysis of the data. The pre and post-inoculation blood glucose levels for ETEC only inoculated calves were very similar, but the post-inoculation levels of Cryptosporidium sp inoculated calves were lower than pre-inoculation levels.

Serology

None of the calves had K99 antibodies in their sera as determined by the ELISA. Total IgG levels greater than 9mg/ml were present in the sera of all calves tested except calf E38.

DISCUSSION

Conventional calves did not develop diarrhoea when inoculated with ETEC strain B44 at 9 days of age and only 2 of 4 calves became

Table 6.6 Lactase levels at 3 sites in the small intestine
of calves inoculated with ETEC and Cryptosporidium sp

| Enteropathogen inoculated | Calf number | lactase activity in μ moles/min/g wet weight in | | |
|---------------------------------------|----------------|--|---------|-------------|
| | | jejunum | mid-gut | upper ileum |
| None | F 659 | NT | NT | NT |
| | F 697 | >18.5 | >18.5 | 7.8 |
| ETEC | F 692 | >18.5 | >18.5 | >18.5 |
| | F 693 | >18.5 | >18.5 | 6.7 |
| | E236 | >18.5 | >18.5 | 18.5 |
| | E237 | >18.5 | >18.5 | 5.1 |
| <u>Cryptosporidium</u> sp | F 682 | >18.5 | >18.5 | 0 |
| | F 684 | >18.5 | >18.5 | 1.1 |
| | E33 | 14.0 | 8.1 | 4.9 |
| | E34 | 4.4 | 1.8 | 4.4 |
| ETEC and <u>Cryptosporidium</u> sp | E38 | 10.9 | 6.7 | 2.86 |
| | E39 | >18.5 | >18.5 | 5.96 |

NT; not tested

Table 6.7 Lactose tolerance of calves inoculated with ETEC
or Cryptosporidium sp

| Time sample taken (minutes) | blood glucose concentration expressed as a percentage of the starting value in | | | |
|--------------------------------------|---|----------------------|--|----------------------|
| | ETEC inoculated calves (F692 and F693) | | <u>Cryptosporidium</u> sp inoculated calves (F682 and F684) | |
| | Pre- inoculation | Post- inoculation | Pre- inoculation | Post- inoculation |
| 0 | 100 | 100 | 100 | 100 |
| 10 | 130 | 118 | 117 | 80 |
| 20 | 140 | 120 | 151 | 102 |
| 30 | 118 | 115 | 134 | 90 |
| 45 | 115 | 110 | 129 | 82 |
| 60 | 107 | 115 | 165 | 91 |
| 75 | 118 | 136 | 164 | 103 |
| 90 | 140 | 172 | 144 | 115 |
| 120 | 142 | 148 | 117 | 60 |

diarrhoeic when inoculated at a similar age with Cryptosporidium sp. Dual inoculation of calves with Cryptosporidium sp at 9-days-old followed by ETEC at 11 days resulted in the establishment of diarrhoea and Cryptosporidium sp infection in only one calf, while ETEC adherence was not observed in the intestines of either calf. The age of exposure to ETEC and Cryptosporidium sp was selected from previous experiments with ETEC and rotavirus in calves (Tzipori, Makin, Smith and Krautil, 1981).

All 4 ETEC inoculated calves remained clinically normal throughout the 10 to 13 day experimental period. The challenge ETEC strain was detected in faeces 24 hours p.i. and possessed both virulence characters, K99 and STa. Bacterial counts from 7 intestinal sites indicated that coliform levels were lower in all 4 animals compared with levels in the intestine of calf F609, which had been infected with the same ETEC strain under 18 hours of age. These results indicate that although the ETEC strain excreted in the faeces was potentially pathogenic, it was unable to adhere to the small intestine and cause diarrhoea in 9-day-old calves. The mechanism by which calves become resistant in this way is not known. In vitro studies demonstrated that the K12:K99 E. coli strain could adhere to epithelial cell brush borders prepared from a 9-day-old calf (Chapter 4, Section B). This may suggest that resistance is not due to the loss of receptors for K99 antigen, but exclusion factors may be involved in vivo which prevent ETEC from adhering.

Although adherent bacteria were not observed by histological or IF studies, pathological changes were found in the lower ileum of 3 calves inoculated with ETEC. These changes were similar to those

reported in calves inoculated at less than 24 hours of age (Pearson, McNulty and Logan, 1978; Bellamy and Acres, 1979; Hadad and Gyles, 1982a). The similarity of the pathological lesions implies that the cause may be similar but it cannot exclude a different mechanism. Possibly minor lesions may be present in older ETEC infected calves without the induction of diarrhoea.

The pathological changes and clinical symptoms exhibited by the Cryptosporidium sp only infected calves were variable. Calves E33 and E34 have been discussed before (Chapter 5, Section B). Calves F682 and F684 did not show any marked clinical symptoms post-inoculation with Cryptosporidium sp and the pathological changes were much milder than those of clinically-ill calves, E33 and E34. The variation in response of calves F682 and F684 as compared with E33 and E34 could be attributed to strain variations in Cryptosporidium sp, difference in infectivity or titre, or differences in the breeds of calves used.

The combined ETEC/Cryptosporidium sp inoculated group contained only 2 calves, one of which did not become infected with Cryptosporidium sp. The effect of dual infection can therefore only be considered in one calf, thus the lack of objective analysable data prohibits any firm conclusions being made. Although ETEC neither multiplied nor adhered to the small intestine of Cryptosporidium sp infected calf E38, further experiments have to be performed to confirm this result.

The lactase studies indicated that all clinically affected calves (E33, E34 and E38) had decreased lactase levels in the jejunum and mid-gut compared with the clinically normal calves (E236, E237, E39, F682, F684, F692, F693 and F697). Statistical analysis could

not be performed on this data, but they support the presence of mucosal damage as shown by histological findings in clinically affected calves.

The lactose tolerance tests indicate no apparent differences in blood glucose levels before or after inoculation with the ETEC strain, but levels in calves challenged with Cryptosporidium sp fell after inoculation. Although the data could not be analysed statistically, the results suggest that Cryptosporidium sp infection induces a decrease in the digestive and absorptive capacity of the intestine.

In conclusion, only subclinical infections were observed in 9-day-old calves inoculated with ETEC strain B44 and variable clinical responses were observed with Cryptosporidium sp in calves of this age. The infection of calves with ETEC and Cryptosporidium sp could only be assessed in one calf, thus no firm conclusions could be made on this dual infection situation.

CHAPTER 7

GENERAL DISCUSSION

The primary aims of this study were to determine the prevalence of calf ETEC infections in the United Kingdom, in particular Scotland and Northern England, to establish whether the characteristics of ETEC strains isolated were similar to those described in other countries and to initiate studies on the immunisation of pregnant cows with a K99 antigen/rotavirus vaccine.

During the course of the calf diarrhoea survey Cryptosporidium sp was identified in the faeces of diarrhoeic calves (Snodgrass, Angus, Gray, Keir and Clerihew, 1980; Tzipori, Campbell, Sherwood, Snodgrass and Whitelaw, 1980). This led to studies on the biology of Cryptosporidium sp in laboratory mice and on its pathogenicity in SPF and conventional calves and gnotobiotic lambs. Mixed enteric infections were also studied in which conventional calves and gnotobiotic lambs were inoculated with ETEC, Cryptosporidium sp and rotavirus.

The general discussion collates information obtained during the course of this study and suggestions are made for future research which may provide additional useful data.

Survey of ETEC

ETEC infections were detected in diarrhoeic calves of 1 to 3 days of age (Chapter 3) and were present in calves from 11% of the outbreaks studied, a frequency lower than that reported in other countries (Acres, Laing, Saunders and Radostits, 1975; Morin, Lariviere and Lallier, 1976; Isaacson, Schneider and Moon, 1978;

Nakazawa, Nemoto, Ueda and Maruyama, 1981). No previous surveys have been reported in Britain concerning the extent of ETEC associated diarrhoea in the neonatal calf population, although a similar but unreported survey of calves in the south of England has so far failed to attribute any of 12 outbreaks of calf diarrhoea to ETEC (D. Reynolds, Compton, England, personal communication). Support for the opinion that K99⁺ ETEC infections are relatively uncommon was provided by the serological surveys (Chapter 3) in which only 2.9% of calf sera and 3.9% of adult cow sera contained detectable K99 antibodies. Sero-epidemiological studies indicate prevalence of infection but not necessarily of disease, which may occur in only a small proportion of infected animals. Furthermore, antibodies in calf sera may have been either active or maternal in origin, thus giving an overestimate in this respect. The high mortality associated with enterotoxigenic colibacillosis may however counter-balance these overestimates as severely affected calves may die due to infection. One aspect not investigated in the serological survey was the occurrence of K99 antibodies in surviving calves from herds affected with K99⁺ ETEC, although evidence that calves seroconvert to the K99 antigen was provided by the calves from unvaccinated dams (Chapter 4), which were tested at 28 days post challenge with K99⁺ ETEC.

The characteristics of the isolated ETEC strains were similar to those found in other countries (Moon, Whipp and Skartvedt, 1976; Myers and Guinee, 1976; Isaacson, Schneider and Moon, 1978; Kornitzer and Tamarin, 1979; Nakazawa, Nemoto, Ueda and Maruyama, 1981; Libya, Chapter 3). There was complete correlation between possession of

K99 antigen and production of STa; K99⁻ STa⁺ strains were not detected. Other studies in which K99⁻ STa⁺ strains were isolated showed that some produced the 987P pilus antigen (Isaacson, Schneider and Moon, 1978). E. coli isolates were not screened for the recently described F41 antigen (Morris, Thorns, Scott, Sojka and Wells, 1982) which has so far been found only on K99⁺ strains of the 09 and 0101 sero-groups (Morris, Thorns and Sojka, 1980), nor examined for their possible adherence to isolated calf epithelial cell brush borders or intestinal villi in vitro. Clearly these are areas in which further work is necessary.

Inoculation of calf ligated intestinal loops was considered to provide the most accurate assessment of the pathogenicity and enterotoxigenicity of ETEC other than actual oral inoculation of newborn calves. Eight K99⁻ STa⁻ E. coli isolates produced a non-neutralisable, cell-rounding factor in the Y1 adrenal cell assay. It is uncertain whether this factor exerts pathogenic effects in vivo, but the inability of such strains to dilate calf gut loops suggests that they are not virulent. Whether the LT-like factor is active in other ways remains to be examined, but the small number of isolates possessing the characteristic suggests that they are not of much importance in calf diarrhoea.

K99⁺ ETEC isolated during the survey were found to be sensitive to a variety of antibiotics. This is surprising especially when resistance plasmids have been found in 80 to 100% of E. coli isolated from enteric disorders of newborn animals (Jorgensen, 1981) and since it is common practice to treat diarrhoeic calves with oral antibiotics. Although comparisons with other surveys may not be valid because of

differences in sensitivity testing methods, Sivaswamy and Gyles (1976b) reported higher antibiotic resistances than those found in this study. A survey by Jackson (1981) in Britain reported on resistance patterns of E. coli isolated from farm animals; 50% of cattle isolates were from enteric disorders (undescribed) and the antibiotic resistances reported were similar to those in this survey.

Modifications could have been made to the calf diarrhoea survey. Firstly, it would have been desirable to obtain calves from each outbreak for necropsy which could have enabled correlation of enteropathogens with intestinal lesions by histological and immunofluorescence studies (Isaacson, Schneider and Moon, 1978; Moon, McClurkin, Isaacson, Pohlenz, Skartvedt, Gillette and Baetz, 1978). By this method atypical calf enteropathogenic E. coli (K99⁻ and STa⁻) may be found adhering to the intestine. Secondly, the E. coli characterisation procedures were time consuming. Nearly 700 isolates were tested for K99 antigen and STa, the result of which showed complete correlation between the presence of K99 and STa; thus in future, screening need only employ tests for one of these characters. The relative ease with which K99 detection is performed suggests that this method is preferable, but new, easier techniques for STa detection are alternatively available (Frantz and Robertson, 1981; Gianella, Drake and Luttrell, 1981).

Identification of K99⁺ E. coli would provide Veterinary Investigation Centres (V.I.C.) with a rapid diagnosis of ETEC associated calf diarrhoea. During the course of this study, specific K99 anti-serum and details of detection methods were distributed by the author to several V.I.C. laboratories throughout Scotland.

Aspects of Cryptosporidium sp infections

It became apparent that the priorities for research on Cryptosporidium sp were the following: investigation of its significance in calf diarrhoea; establishment of an experimental model for disease; development of a method for standardising and titrating inocula; development of a stable storage method; in vitro culturing of the organism; chemoprophylaxis of susceptible animals; and disinfection of farm premises and laboratories.

A good correlation was obtained for the association of Cryptosporidium sp with calf diarrhoea in a single outbreak (Tzipori, Campbell, Sherwood, Snodgrass and Whitelaw, 1980) and in a survey (Snodgrass, Sherwood, Terzolo and Synge, 1982). In the survey Cryptosporidium sp was found in 20% of diarrhoeic calves, but was the predominant enteropathogen on only 3 farms. Thus, the correlation of Cryptosporidium sp with major diarrhoea outbreaks may only be found in a few instances.

The results of experimental infection of calves and lambs with Cryptosporidium sp (Chapter 5 and 6) agree with those of Moon and Bemrick (1981) and Tzipori, Angus, Gray, Campbell and Allan (1981). The clinical severity of the disease produced in lambs was consistently more severe than that observed in calves of a similar age irrespective of the source of inoculum. Field outbreaks of calf diarrhoea which involved only Cryptosporidium sp were associated with moderate clinical symptoms and low mortality (Tzipori, Campbell, Sherwood, Snodgrass and Whitelaw, 1980; Anderson and Bulgin, 1981). Diarrhoea outbreaks in lambs however were of high morbidity, with a high mortality of 30% (Tzipori, Angus, Campbell and Clerihew, 1981; Angus, Appleyard, Menzies,

Campbell and Sherwood, 1982). However, severe clinical illness and mortality of up to 50% were observed when Cryptosporidium sp was involved with other enteropathogens in calf diarrhoea (Nagy, Antal and Lakner, 1980; Jerret and Snodgrass, 1981). Thus under field conditions mixed infections involving Cryptosporidium sp appear to be more severe than infections of Cryptosporidium sp only.

Suckling mice were evaluated as an experimental model for the disease (Sherwood, Angus, Snodgrass and Tzipori, 1982). Only subclinical infections could be established and no pathological changes were observed in the intestines of infected mice. This is in contrast to clinical infections observed in calves and lambs (Chapters 5 and 6). However, this model proved useful for titration of the infectivity of experimental inocula, and allowed their standardisation between experiments. This system was also used to investigate the storage of Cryptosporidium sp in laboratory media. Freezing was found to destroy the viability of the organism in this and another study (Heine and Boch, 1981). In contrast Nagy, Antal and Lakner (1980) reported that gut scrapings remained infective after storage at -15°C for 1 to 2 months, although it is possible that the calves which they used were naturally infected with Cryptosporidium sp. Cryptosporidium sp was found to remain viable for 4 to 6 months if stored at 4°C in PBS or 2.5% potassium dichromate, but even under these conditions viability rapidly decreased. A stable storage system still has to be found, until which time it is recommended that the infectivity of inocula should be confirmed shortly before and during an experiment.

The in vitro culturing of Cryptosporidium sp was not investigated in this study although tissue culture systems have been devised for the growth of coccidia such as Eimeria spp (Todd and Ernst, 1977) and could be modified for the growth of Cryptosporidium sp. Workers in the USA have developed a technique for growth of the organism in chick eggs, but so far this information has not been reported. Tissue cultures could be used to isolate Cryptosporidium sp from faecal bacteria although bacteria free faecal suspensions of the organism have been prepared by treatment of experimentally infected gnotobiotic lambs with antibiotics (Snodgrass, Angus and Gray, unpublished results) or of infected faeces with 60% alcohol (Tzipori, Smith, Makin and Halpin, 1982).

Coccidial infections have been successfully controlled by the use of sulphamezathine or other sulphonamides in cattle, and of nitrofurazone, amprolium or decoquinate in poultry (Todd and Ernst, 1977). So far over 40 anti-microbial agents, including the above mentioned coccidiostats, have been tested against cryptosporidiosis of humans, calves or experimentally infected mice, without success (reviewed by Tzipori, 1982). Further experiments are required to find a chemotherapeutic agent which may prevent cryptosporidiosis. At present, the mouse model is being used as a screening procedure for the action of various drugs.

The only disinfectants known to be effective against Cryptosporidium sp at present are formalin and household ammonia (Campbell, Tzipori, Hutchison and Angus, 1982). Two aldehyde-based disinfectants proved to be ineffective (Angus, Sherwood, Hutchison and Campbell, 1982) and further investigations are clearly needed to identify other effective but more practical disinfectants.

Cryptosporidium sp is a member of the suborder Eimeriorina, which includes recognised pathogenic 'coccidia' such as Eimeria spp, Isospora spp, Toxoplasma spp and Sarcocystis spp (Todd and Ernst, 1977). These coccidia are larger than Cryptosporidium sp, are found deep in the cytoplasm of cells and in general are species specific. In contrast, the evidence presented in Chapter 5 on the passage of calf Cryptosporidium sp in lambs and mice and studies by Heine and Boch (1981), Moon and Bemrick (1981), Tzipori and Campbell (1981), Brandler (1982) and Reese, Current, Ernst and Bailey (1982) suggest that Cryptosporidium sp is not species specific. This evidence supports the concept of a single-species genus suggested by Tzipori, Angus, Campbell and Gray, (1980) and has particular significance for Cryptosporidium sp infections in man, as normal immuno-competent humans have developed infections after contact with Cryptosporidium sp infected calves (Anderson, Donndelinger, Wilkins and Smith, 1982; Anonymous, 1982; Current and Reese, 1982). Thus Cryptosporidium sp is a potential zoonosis.

Other enteric infections

Some calves in the diarrhoea surveys had mixed enteric infections. ETEC/rotavirus infection was observed in 3 of 23 ETEC infected calves in the U.K. and in 3 of 7 ETEC infected calves in Libya. Surveys in other countries have demonstrated up to 50% of diarrhoeic ETEC infected calves to be concurrently infected with rotavirus (Acres, Laing, Saunders and Radostits, 1975; Moon, McClurkin, Isaacson, Pohlenz, Skartvedt, Gillette and Baetz, 1978). Experimental studies have indicated a possible interaction between these two agents (Gouet, Contrepois, Dubourguier, Riou et al, 1978; Runnels, Moon, Whipp,

Matthews and Woode, 1980; Tzipori, Makin, Smith and Krautil, 1981; Snodgrass, Smith and Krautil, 1982) although the exact mechanism of interaction was not clarified.

Mixed infection experiments were studied in 4 to 7-day-old lambs using ETEC and rotavirus (Chapter 6; Tzipori, Sherwood, Angus, Campbell and Gordon, 1981). Clinical infections were not established which is in contrast to the clinical infections observed in the calf experiments cited above. The differences between the results of the present studies in lambs and those obtained previously in calves may be due to the enteropathogenicity of the agents used, variation in the susceptibility between calves and lambs or differences in experimental design. Runnels, Moon, Whipp, Matthews and Woode (1980) demonstrated ETEC adherence in the small intestine of 5 to 8-day-old gnotobiotic calves previously infected with rotavirus, but no adherence in control calves. ETEC adherence was not observed in the lamb experiments although the ETEC challenge strain was excreted until necropsy.

Beachey (1981) cited work in which Pseudomonas aeruginosa adhered more avidly to mouse tracheal epithelial cells in vitro if the cells were pre-infected with influenza virus. Although the mechanism of increased adherence was unclear it was postulated that, in addition to native receptors on host cells, new receptors may have been generated by virus induced changes. This concept may be applicable to the ETEC/rotavirus situation where rotavirus infection could generate an increase in receptor density for the adhesive antigens on ETEC. In vitro studies with isolated calf intestinal epithelial cells from normal and rotavirus infected calves may help to identify the mechanism of interaction between ETEC and rotavirus.

Studies using gnotobiotic lambs and conventional calves indicated an age related resistance to ETEC infections which has been observed before (Smith and Halls, 1967a). The mechanism by which this resistance develops is not known, but in vitro studies by Runnels, Moon and Schneider (1980) demonstrated a gradual reduction in adherence of K99⁺ E. coli to intestinal epithelial cells isolated from calves between 12 hours and 2 weeks of age. This suggests that receptors for the K99 antigen in calves decrease in density with age. This is in direct contrast to the findings in Chapter 4 where K12: K99 E. coli adhered to brush borders prepared from intestinal epithelial cells of a 9-day-old calf, an age at which in vivo adherence was not observed (Chapter 6). This may indicate that in vivo resistance to adherence may be due to exclusion factors at the mucosal surface rather than a decrease in K99 antigen receptor sites on epithelial cells. Further work is clearly needed to identify the mechanism by which in vivo development of resistance to ETEC adherence develops in calves and lambs; an insight to these mechanisms may provide useful data on the interaction of ETEC and rotavirus.

Dual ETEC and Cryptosporidium sp infections have been reported in outbreaks of calf diarrhoea (Nagy, Antal and Lakner, 1980; Snodgrass, Angus, Gray, Keir and Clerihew, 1980). The mixed infection experiments in conventional calves reported in Chapter 6 require repetition due to the small number of calves in the ETEC/Cryptosporidium sp challenge group. Findings from the limited number of gnotobiotic lambs challenged with ETEC and Cryptosporidium sp suggested that Cryptosporidium sp infection did not predispose the animals to ETEC adherence, although

ETEC were excreted in the faeces until necropsy. Furthermore, subjective assessment suggested that clinical signs in the ETEC/Cryptosporidium sp challenge group appeared to be of equal severity to the Cryptosporidium sp group.

Vaccination against ETEC and rotavirus

The multifactorial infectious nature of neonatal calf diarrhoea has hindered attempts to produce an effective broad-spectrum vaccine, and the efficacy of commercial vaccines has on occasion been questioned (de Leeuw, Ellens, Talman, Zimmer and Kommrij, 1980; Myers and Snodgrass, 1982). The work presented in Chapter 4 suggested that the trial K99/rotavirus vaccine protected calves against experimental enterotoxigenic colibacillosis; its efficacy against rotavirus challenge was not assessed. Antibody response to the rotavirus component has been examined before (Snodgrass, Fahey, Wells, Campbell and Whitelaw, 1980). Although calves were not protected against diarrhoea after rotavirus challenge, there was a significant delay in the onset of diarrhoea, and a decreased duration of rotavirus excretion in calves from vaccinated dams compared with those from unvaccinated dams. It was considered that the rotavirus challenge may have been high enough to swamp the neutralising effect of passively acquired rotavirus antibody.

The K99/rotavirus vaccine must be shown to be effective in field trials before it can be commercially produced. Trials to date suggest that the vaccine reduces the occurrence of rotavirus associated diarrhoea in calves from vaccinated dams, but it has not been possible to assess the effectiveness of the K99 component as no K99⁺ ETEC associated outbreaks have occurred on trial farms.

The use of the K99 component (and F41) extracted from strain B41 is unlikely to protect against strains carrying other pilus antigens such as 987P (Isaacson, Schneider and Moon, 1978) or against polysaccharide antigens (Smith and Huggins, 1978; Hadad and Gyles, 1982b) which may play a significant role in the adherence of ETEC to calf small intestine. However, in Britain such atypical calf ETEC strains have not been detected so far (Chapter 3, N. Chanter and J. Morris, personal communication). The use of B44 as the challenge ETEC strain in the vaccine trial (Chapter 4) suggests that the K30 antigen, previously reported to be a possible adhesive antigen (Smith and Huggins, 1978) did not allow significant adherence in calves from vaccinated dams.

Further improvement of the vaccine could be afforded by the inclusion of a coronavirus component. However, the survey data (Chapter 3) suggests that this enteropathogen is of very low occurrence in Britain, although it has been more frequently isolated from diarrhoeic calves in other countries (Morin, Lariviere and Lallier, 1976; Moon, McClurkin, Isaacson, Pohlenz, Skartvedt, Gillette and Baetz, 1978).

Recently, neutralisation tests have suggested that there are serologically distinct calf rotaviruses in Britain (Snodgrass and Ojeh, unpublished data). The strain of calf rotavirus used in the present vaccine is the same serotype as 95% of the field isolates investigated so far (Snodgrass and Ojeh, unpublished data). The use of one particular serotype in a vaccine may cause the emergence of a serologically distinct rotavirus capable of evading the protective effects of the vaccine. Thus, the vaccine may in future require

modification by inclusion of other rotavirus serotypes; constant surveillance will be necessary to identify the predominant rotavirus serotypes present in the U.K. calf population.

The oil vaccine used in Chapter 4 seemed to have 2 major advantages over the Alhydrogel vaccine. Firstly, one dose only was required to initiate a high serum antibody response in the pregnant cow. The level and persistence of this response may mean that cows need to be vaccinated only once every two years. Secondly, the prolonged secretion of antibody in milk for 7 to 14 days post partum suggests that the vaccine may be able to protect against dual K99⁺ ETEC/rotavirus infections of young calves. These aspects have to be verified by further investigations on the vaccine.

To conclude, the major points arising from this work are: ETEC is a major cause of diarrhoea in calves under 2 days of age although diarrhoea in this age group is less common than rotavirus associated diarrhoea in older animals; ETEC strains isolated from calves in Britain were K99⁺ STa⁺ and dilated calf ligated gut loops, which is in agreement with work from other countries; epidemiological and experimental work established the rationale for the development of a K99 antigen/rotavirus vaccine; and Cryptosporidium sp can be a primary cause of diarrhoea in calves (and lambs), but its role in field outbreaks of diarrhoea has to be further assessed.

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Appendix 1. K99 antibody titres in the sera of steers
before vaccination in Experiment 2 as
measured by the sandwich ELISA

| Group | Steer (\log_{10} K99 antibody titre) | | | | | | | | | |
|-------|---|-----|-----|-----|-----|-----|-----|-----|---|-----|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| 1 | - | 2.6 | - | 2.4 | 2.6 | 2.5 | 2.1 | 2.2 | - | 2.2 |
| 2 | - | - | - | - | 2.4 | 2.6 | - | - | - | - |
| 3 | 2.3 | 2.3 | 2.3 | - | 2.2 | | | | | |

Appendix 2. Faecal excretion of ETEC strain B44 6 days after challenge of calves

| Vaccine Group | % faecal excretion over 6 days post challenge for calves as measured by slide agglutination using either anti-K99 or anti-B 44 serum | | | | | | | | | | | |
|---------------------|--|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| | 1 | | 2 | | 3 | | 4 | | 5 | | 6 | |
| | K99 | B44 | K99 | B44 | K99 | B44 | K99 | B44 | K99 | B44 | K99 | B44 |
| Control | 100 | 100 | NT | NT | 90 | 89 | 83 | 83 | 47 | 61 | 80 | 72 |
| 2ml oil adjuvant | 80 | 92 | 47 | 78 | 77 | 89 | 10 | 22 | 77 | 89 | 33 | 11 |
| 0.5ml oil adjuvant | 0 | 47 | 40 | 61 | 23 | 50 | 8 | 59 | 0 | 0 | 27 | 33 |
| Alhydrogel adjuvant | 0 | 33 | 40 | 50 | 28 | 53 | 50 | 67 | 13 | 39 | 53 | 50 |

APPENDIX 3. Titration of Cryptosporidium sp (inocula
5 and 6) after storage in laboratory media
at different temperatures

Storage at 37 °C

| Inoculum | Medium | Time of storage (hours) | | | |
|----------|--------|-------------------------|------|-------------------|-----|
| | | Log ₁₀ | | MID ₅₀ | |
| | | 2 | 4 | 8 | 120 |
| 6 | PBS | 3.08 | 2.33 | 2.20 | - |

Storage at room temperature

| Inoculum | Medium | Time of storage (days) | | | | | |
|----------|--------|------------------------|-----|-----|-------------------|-----|-----|
| | | Log ₁₀ | | | MID ₅₀ | | |
| | | 2 | 7 | 27 | 29 | 58 | 100 |
| 5 | PBS | NT | 5.0 | 3.5 | NT | 1.0 | - |
| 6 | PBS | 3.08 | NT | NT | - | NT | NT |

-; MID₅₀<1.0.

NT; not tested.

APPENDIX 3. continued

| Storage at 4 °C | | Log ₁₀ MID ₅₀ | | | | | | | | | | | | | |
|-----------------|---------------------------|-------------------------------------|-----|----------|----------|----------|----------|----------|----------|----------|-----------|------------|------------|--|--|
| Inoculum Medium | | Time of storage (days) | | | | | | | | | | | | | |
| | | 2 | 7 | 31 to 35 | 36 to 40 | 41 to 45 | 46 to 55 | 56 to 65 | 76 to 85 | 86 to 95 | 96 to 105 | 125 to 135 | 180 to 190 | | |
| 5 | PBS | NT | 5.0 | NT | NT | NT | 3.9 | NT | 4.36 | NT | 4.16 | 3.2 | 2.38 | | |
| 5 | 2.5% potassium dichromate | NT | 5.0 | 4.56 | NT | NT | NT | 3.8 | NT | NT | NT | 4.3 | 3.5 | | |
| 6 | PBS | 3.08 | NT | NT | NT | 3.08 | NT | NT | 2.87 | NT | - | | | | |
| 6 | 5% BSA | 3.08 | NT | 3.03 | NT | NT | NT | 2.37 | - | | | | | | |
| 6 | distilled water | 3.08 | NT | NT | 1.92 | NT | - | | | | | | | | |
| 6 | 2.5% potassium dichromate | 3.08 | NT | NT | 2.51 | NT | NT | 3.12 | NT | 3.08 | NT | 2.29 | - | | |

-; MID₅₀<1.0.
NT; not tested.

Appendix 4. Sucrase, lactase and maltase activity (μ moles/min/g wet weight) in the small intestine of infected and uninfected lambs

| Group ^a | Lamb number | enzyme activity in μ moles/min/g wet weight in | | | | | | | | | | | |
|-------------------------------|---------------------|--|-------|------|---------|-------|------|-------------|------|------|------|------|------|
| | | duodenum | | | mid gut | | | lower ileum | | | | | |
| | | S | L | M | S | L | M | S | L | M | S | L | M |
| 1 (subclinically infected) | 6 | 0.34 | 5.25 | 0.17 | 0.54 | 3.80 | 0.05 | 0.81 | ND | ND | 0.81 | ND | ND |
| | 14 | 1.87 | 10.86 | NT | NT | 8.82 | 0.23 | NT | 0.52 | 0.29 | NT | 0.52 | 0.29 |
| | 15 | 0.83 | 7.34 | ND | NT | 6.11 | ND | ND | 1.71 | 0.46 | ND | 1.71 | 0.46 |
| | 16 | 0.35 | 3.33 | 0.85 | 0.15 | 13.26 | 0.50 | 0.08 | 1.5 | 0.78 | 0.08 | 1.5 | 0.78 |
| 2 (clinically infected) | 18 | 0.13 | 3.47 | 0.93 | ND | 2.06 | 0.77 | 0.07 | ND | 0.1 | 0.07 | ND | 0.1 |
| | 19 | ND | 1.48 | ND | ND | 0.60 | ND | 0.24 | 0.91 | ND | 0.24 | 0.91 | ND |
| | 20 | ND | 0.47 | 0.76 | ND | 1.32 | 0.43 | ND | 0.17 | 0.25 | ND | 0.17 | 0.25 |
| | 21 | 0.08 | 3.56 | 0.70 | ND | 0.62 | 0.49 | ND | ND | 0.5 | ND | ND | 0.5 |
| | 22 | 1.96 | 1.59 | 0.18 | 0.85 | 1.17 | 0.13 | 0.13 | 0.3 | 0.28 | 0.13 | 0.3 | 0.28 |
| 3 (control) | 6 days ^b | NT | 5.98 | NT | NT | 6.70 | NT | NT | 7.92 | NT | NT | 7.92 | NT |
| | 7 days | ND | 4.60 | 0.46 | 2.2 | 2.3 | 0.46 | ND | ND | ND | ND | ND | ND |
| | 10 days | NT | 3.95 | NT | NT | 2.29 | NT | NT | 0.62 | NT | NT | 0.62 | NT |
| | 13 days | 0.47 | 5.47 | 0.41 | 1.63 | 4.83 | 0.56 | 2.6 | 0.50 | 0.57 | 2.6 | 0.50 | 0.57 |
| | 17 days | 1.18 | 3.25 | 0.23 | ND | 4.88 | 0.92 | ND | 0.23 | 0.59 | ND | 0.23 | 0.59 |

a; information on grouping of gnotobiotic lambs is given in the results section for enzymology (Chapter 6, Section A)

b; days of age when killed

NT; not detected

NT; not tested
S; sucrase
L; lactase
M; maltase

PUBLICATIONS ARISING FROM THIS THESIS

Angus, K.W., D. Sherwood, G. Hutchison and I. Campbell. (1982). Evaluation of the effect of 2 aldehyde-base disinfectants on the infectivity of faecal cryptosporidia for mice. *Research in Veterinary Science*. 33 : 379-381.

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Tzipori, S., D. Sherwood, K.W. Angus, I. Campbell and M. Gordon. (1981). Diarrhoea in lambs : Experimental infections with enterotoxigenic Escherichia coli, rotavirus and Cryptosporidium. *Infection and Immunity*. 33 : 401-406.

An outbreak of calf diarrhoea attributed to cryptosporidial infection

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Veterinary Record (1980) 107, 579-580

Coccidian parasites of the genus *Cryptosporidium* have been isolated from several hosts including mammals, birds and reptiles (Levine 1973) and probably have a world wide distribution. Cryptosporidia are extracellular organisms infecting the lower small intestine (Vetterling and others 1971, Barker and Carbonell 1974, Pohlenz and others 1978). Although their occurrence was reported by Tyzzer in 1907, only recently has their association with diarrhoea in calves (Meuten and others 1974, Pohlenz and others 1978, Snodgrass and others 1980a) and lambs (Berg and others 1978) been described. The significance of calf cryptosporidiosis has been difficult to assess in the cases reported (Pohlenz and others 1978, Snodgrass and others 1980a) because of the presence of other enteropathogens. A natural outbreak of calf diarrhoea in which cryptosporidia, the only enteropathogens detected, were probably the primary cause is reported here.

The outbreak occurred in a housed beef herd of 41 blue-grey sucking calves, born over a period of eight weeks. The herd, which had previous history of severe neonatal diarrhoea, was involved in a vaccination experiment. Half the cows (21) were vaccinated before calving with experimental calf rotavirus vaccine described by Snodgrass and others (1980b). Faeces were collected from each of the 41 calves on the sixth and 14th day after birth. Twenty-eight days after the first calving, when diarrhoea first started, faeces were also collected from scouring calves at the onset of the clinical illness. A total of 101 faecal samples was screened (62 normal, 39 diarrhoeic) for bovine rotavirus and coronavirus by enzyme-linked immunosorbent assay (ELISA) tests (Ellens and de Leeuw 1977), for other enteric viruses by electron microscopy (Snodgrass and others 1976) and for K99 possessing *Escherichia coli* (Moon and others 1976). Oocysts of cryptosporidium were observed in faecal smears stained with Giemsa (Pohlenz and others 1978).

Table 1 summarises data collected from the affected herd. Fig 1b shows the pattern of the outbreak in terms of the number of scouring calves on days after the first calving. The total number of calves present on certain days is indicated. The distribution of scouring calves follows a near normal curve including a smaller pattern on the right (at 60 days) which represents a relapse in eight of the 35 scouring calves. Only six calves escaped diarrhoea. Fig 1a shows the number and distribution of faecal samples collected on the first day of diarrhoea from each of the 31 scouring calves tested and the proportion in which oocysts could be detected. In the 62 normal faecal samples neither oocysts, enteric viruses nor enterotoxigenic *E. coli* were detected.

Generally, the calves experienced a mild to moderate degree of illness with occasional signs of anorexia and depression among the younger animals. Affected calves were treated daily with kaolin and pectin (Kaogel; Park Davis) and a solution of electrolytes, glycine and dextrose (Ionaid; Syntex Pharmaceuticals); kaolin and pectin and a solution of electrolytes and dextrose (Electrosol; Willington Medicals); kaolin and pectin and saline; or a sulphadiazine, sulphamerazine and sulphapyridine formulation (Trinamide; May & Baker), depending on age and severity of diarrhoea.

Positive diagnosis of cryptosporidiosis in calves has in the past been confirmed by the demonstration of parasites

TABLE 1: Summary of data collected from a suckler beef herd with suspected cryptosporidiosis

| | |
|---|---------------------|
| Number of calves in the herd | 41 |
| Number of faecal samples examined | 101 |
| Number of calves with diarrhoea | 35 (85%) |
| Number of calves with diarrhoea tested | 31 |
| Number of calves shedding oocysts | 18 (60%) |
| Number of calves with recurrence of diarrhoea | 8 (23%) |
| Number of calves with recurrence and shedding oocysts | 4 |
| Number of normal faeces containing oocysts | 0 |
| Youngest calf with diarrhoea (days) | 5 |
| Youngest calf shedding oocysts (days) | 6 |
| Oldest calf with diarrhoea (days) | 39 |
| Oldest calf shedding oocysts (days) | 26 |
| Mean age of onset of diarrhoea (days) | 15.3 (± 1.15) |
| Mean interval between two episodes of diarrhoea | 7.1 (± 1.51) |
| Mean duration of first episode (days) | 7.7 (± 0.73) |
| Mean duration of second episode (days) | 4.4 (± 0.60) |
| Age when recurrence began (days) | 16.5 (± 1.81) |
| Longest duration of diarrhoea (days) | 16 |
| Correlation between age and duration of diarrhoea | Not significant |
| Correlation between age and recurrence of diarrhoea | Not significant |

(\pm Standard error)

attached to the brush borders of the enterocytes. Since none of the calves died and no histopathological material was available we resorted to experimental inoculation of a calf which had been previously exposed to the most commonly encountered enteropathogens to eliminate the risk of natural infections during the experiment. A 10 ml suspension (10 per cent v/v) prepared from faeces containing oocyst was fed to a seven-day old colostrum-fed calf which had fully recovered from a previous dual infection with bovine rotavirus and enterotoxigenic *E. coli* (Tzipori and Campbell unpublished data).

Nine days after inoculation the calf developed moderate diarrhoea with coincident shedding of oocysts in the faeces, at which time it was killed. Examination of haematoxylin and eosin stained gut sections revealed numerous cryptosporidium attached to the brush borders of the intestinal enterocytes. The faeces and gut contents were screened for other enteropathogens by methods described above and by immunofluorescent staining. No other agent was detected in this calf throughout the incubation period or at post mortem examination.

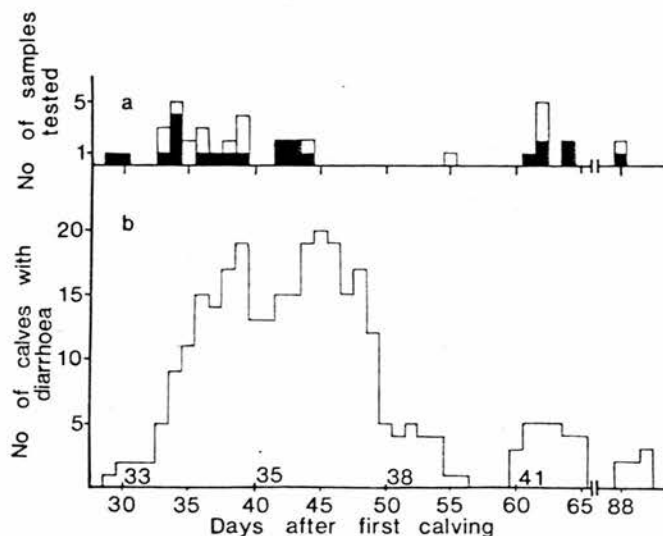


FIG 1: (a) Number and distribution of faecal samples collected on the first day of scouring from 31 calves (plus eight relapses) and the proportion which contained cryptosporidial oocysts (solid columns); (b) number of scouring calves for each day following first calving (numbers below the horizontal axis) and the number of calves present on certain days (numbers above the horizontal axis)

In this outbreak of calf diarrhoea cryptosporidium was the only demonstrable cause. The disease occurred in calves aged one week and older probably because of the relatively longer incubation period of the cryptosporidium compared to other reported enteropathogens affecting very young animals. The duration of the disease was also longer and calves usually scoured intermittently for at least two weeks. In a number of instances calves experienced a second episode of the disease one to three weeks later. Recurrence of diarrhoea following treatment has also been observed in scouring lambs with suspected cryptosporidiosis (Berg and others 1978). Regular treatment of the infected calves with antimicrobial agents may have modified the severity of the disease and could have reduced the number of oocysts shed.

It seems that cryptosporidium acting alone in this outbreak produced only mild to moderate diarrhoea without mortality. The significance of cryptosporidiosis in terms of its contribution to the enteric syndrome in young animals and its prevalence remains to be determined but from the observations reported must be considered in outbreaks of diarrhoea in calves.

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Diarrhea in Lambs: Experimental Infections with Enterotoxigenic *Escherichia coli*, Rotavirus, and *Cryptosporidium* sp.

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Thirteen gnotobiotic lambs, aged from a few hours to 8 days, were inoculated orally with single infections of enterotoxigenic *Escherichia coli* (ETEC) (four animals), lamb rotavirus (five animals), and *Cryptosporidium* (four animals). Six gnotobiotic and two specific-pathogen-free lambs were co-inoculated with either rotavirus and ETEC (four animals), rotavirus and *Cryptosporidium* (two animals), or ETEC and *Cryptosporidium* (two animals). Lambs 4 days of age and older became only subclinically infected with either rotavirus, ETEC (08:K87:K99 ST⁺), or both enteropathogens given simultaneously. Six-day-old lambs inoculated with *Cryptosporidium* became extremely depressed, anorectic, and had intermittent diarrhea. There was no difference in the clinical manifestations, level of disaccharidase activity in the small intestine, or extent of histological damage between lambs inoculated with *Cryptosporidium* alone or together with either of the other two agents. The results indicate that under the conditions of these experiments, lambs become clinically resistant to infection with ETEC, rotavirus, or both agents together, by 4 days after birth, whereas lambs 2 days old or younger were clinically susceptible to infection by these agents. In contrast, they remained clinically susceptible to infection with *Cryptosporidium* up to at least 6 days of age. *Cryptosporidium* infections were not aggravated by coinfection with either ETEC or rotavirus.

Neonatal diarrhea in lambs is considered an important cause of lamb mortality (18). Enteropathogens known to be associated with the disease include enterotoxigenic *Escherichia coli* (ETEC) (2, 22), rotavirus (10, 21), *Cryptosporidium* (3, 23), and possibly astrovirus (20). ETEC are the cause of colibacillosis in many other species and are the subject of many publications (9, 13, 17, 20).

Under field conditions diarrhea in calves (1, 14) tends to be more commonly associated with mixed infections of two or more enteropathogens, but the situation in lambs is unclear and merits investigation. Experimental coinfections with ETEC and rotavirus have been studied in calves (6, 7, 15, 24); the results of these investigations point to the existence of a mechanism of interaction between the two organisms, but the nature and exact site of the interaction remains undefined.

This communication describes clinical and pathological manifestations in gnotobiotic lambs of experimental coinfections involving ETEC, lamb rotavirus, and a calf-derived *Cryptosporidium*.

MATERIALS AND METHODS

Experimental animals. Of 21 Caesarean-derived lambs, 19 were maintained under gnotobiotic conditions in plastic isolators and 2 were maintained under specific-pathogen-free (SPF) conditions. All were fed reconstituted evaporated cows' milk three times daily for the first 3 days and twice daily thereafter. The SPF lambs were kept in an isolated sterile room with no contact with other animals.

Microbiology. (i) ETEC. Strain S13 (08:K87:K99), obtained from H. Williams Smith (Poultry Research Station, Houghton, England), was used in this experiment. The organism produced heat-stable toxin (as tested by the infant mouse assay [5]) but not heat-labile toxin in the Y1 adrenal cell culture test (16). The presence of K99 antigen (K99⁺) was demonstrated by slide agglutination (8). The inoculum used for lamb infection was 2 ml of Trypticase soy broth (BBL Microbiology Systems) containing 10⁸ (lamb no. 2) or 10¹⁰ viable organisms per ml.

Fecal shedding of strain S13 was demonstrated by testing for K99⁺ in ten randomly selected colonies from fecal swab cultures plated on sheep blood agar and MacConkey agar plates (8).

(ii) Rotavirus. Fecal filtrate (20% [vol/vol] in water) of the sixth passage of lamb rotavirus in gnotobiotic lambs (21) was used to inoculate lambs, each

receiving 2 ml orally. Rotavirus in the feces of infected lambs was demonstrated by direct electron microscopy (20) and enzyme-linked immunosorbent assay.

(iii) *Cryptosporidium*. The organism, obtained originally from a calf, was passaged once in newborn suckling SPF rats and twice in SPF lambs (S. Tzipori, K. W. Angus, E. W. Gray, I. Campbell, and F. Allan, Am. J. Vet. Res., in press). A 2-ml homogenate of gut contents (20% [vol/vol] in phosphate-buffered saline) prepared from the second SPF lamb passage was used to inoculate each lamb. The homogenate was examined as described above and found free of enteric viruses and ETEC. Fecal shedding of *Cryptosporidium* oocysts was demonstrated by Giemsa-stained smears.

Inoculation of lambs: single agents. Thirteen lambs up to 8 days of age were inoculated with ETEC (four lambs), rotavirus (five lambs), or *Cryptosporidium* (four lambs) as detailed in Table 1.

Inoculation of lambs with mixed infections. Three groups of lambs aged from 4 to 8 days were inoculated with either ETEC and rotavirus (four lambs), rotavirus and *Cryptosporidium* (two lambs), or ETEC and *Cryptosporidium* (two lambs) (Table 1). As controls, five uninoculated age-matched lambs were used.

TABLE 1. Response of gnotobiotic and SPF lambs to oral inoculations with ETEC, lamb rotavirus, and *Cryptosporidium*, singly or in coinfections

| Lamb no. | Age at inoculation (days) with: | | | Clinical illness ^a | Shedding of organism ^a | Age of lamb at necropsy (days) |
|-----------------|---------------------------------|-----------|------|-------------------------------|-----------------------------------|--------------------------------|
| | <i>Cryptosporidium</i> | Rotavirus | ETEC | | | |
| 1 ^b | — ^c | — | — | — | — | 3–13 |
| 2 ^d | — | — | <1 | + | + | <1 |
| 3 | — | — | 5 | — | + | 8 |
| 4 | — | — | 5 | — | + | 10 |
| 14 | — | — | 8 | — | + | 12 |
| 9 | — | 1 | — | + | + | 4 |
| 10 | — | 2 | — | + | + | 5 |
| 11 | — | 7 | — | — | + | 19 |
| 15 | — | 8 | — | — | + | 10 |
| 16 | — | 8 | — | — | + | 12 |
| 12 | 1 | — | — | + | + | 4 |
| 13 | 1 | — | — | + | + | 6 |
| 17 ^e | 6 | — | — | + | + | 8 |
| 18 | 6 | — | — | + | + | 10 |
| 5 | — | 4 | 5 | — | + | 8 |
| 6 | — | 4 | 5 | — | + | 10 |
| 7 ^f | — | 7 | 7 | — | + | 15 |
| 8 ^f | — | 7 | 7 | — | + | 16 |
| 19 | 6 | — | 8 | + | + | 10 |
| 20 | 6 | — | 8 | + | + | 12 |
| 21 | 6 | 8 | — | + | + | 10 |
| 22 | 6 | 8 | — | + | + | 12 |

^a Symbols: +, positive; —, not detected.

^b Consists of five uninoculated control lambs.

^c —, None.

^d Lamb no. 2 was infected at 6 and killed at 18 h of age.

^e Lamb no. 17 died as a result of infection.

^f Lambs no. 7 and 8 were SPF.

Clinical observations. The lambs were observed daily for clinical signs of illness. When considered necessary some lambs had their milk intake measured. Fecal samples were examined daily for excretion of enteric viruses, ETEC, and *Cryptosporidium* by the methods shown above.

Necropsy. Under terminal anesthesia, representative pieces of intestine were taken from: duodenum, upper and lower jejunum, upper and lower ileum, spiral colon, and cecum. From each sample, portions were taken into 10% formal saline for histology, and duplicate samples were frozen and stored at -20°C for immunofluorescence (IF) and enzymology. Mucosal scrapings were taken from all sites for bacterial counts.

IF. Cryostat sections of lambs infected with ETEC or rotavirus or both were tested with hyperimmune rabbit sera raised against ETEC S13 or rotavirus. Fluorescein-conjugated sheep anti-rabbit antiserum was used as indicator for the indirect IF test.

Enzymology. Small portions of small intestine were analyzed for lactase, maltase, and sucrose activity by the technique described by Dahlqvist (4). Data were analyzed by analysis of variance.

Bacterial counts. Serial, tenfold dilutions of 0.1 g of mucosal scrapings from each intestinal location were plated on MacConkey agar, and viable counts were carried out (12).

RESULTS

Inoculation with a single agent. (i) ETEC. Lamb no. 2, inoculated 6 h after birth with 10^8 organisms, developed profuse diarrhea within 12 h, shed the organism, and was killed 18 h after birth (Table 1). The other three lambs, no. 3, 4, and 14, inoculated with 2×10^{10} organisms when 5 to 8 days old, failed to show any clinical illness although they excreted ETEC in their feces. No decrease in milk intake was observed for lambs no. 3 and 4 (Fig. 1).

Histological examination of the intestines of the above animals revealed pathological changes only in lamb no. 2. The duodenum and upper jejunum of this lamb appeared normal, but throughout the rest of the small intestine, bacteria were seen adhering to the brush borders of villous enterocytes. Pathological changes included hyperemia of mucosal vessels, distention of lacteals, and infiltrates of neutrophils. Small deposits of fibrin-like material were occasionally observed at the base of the mucosa, and limited cytolysis of lymphocytes was seen in the Peyer's patches of the terminal ileum. The colon and cecum were unaffected.

Strain S13-specific fluorescence was seen only in lamb no. 2. Bacteria were seen coating villi from the upper jejunum to the terminal ileum, indicating surface colonization by the ETEC strain throughout the greater part of the small intestine.

(ii) Rotavirus. Five lambs (no. 9–11, 15, 16) were inoculated with rotavirus (Table 1). Lambs

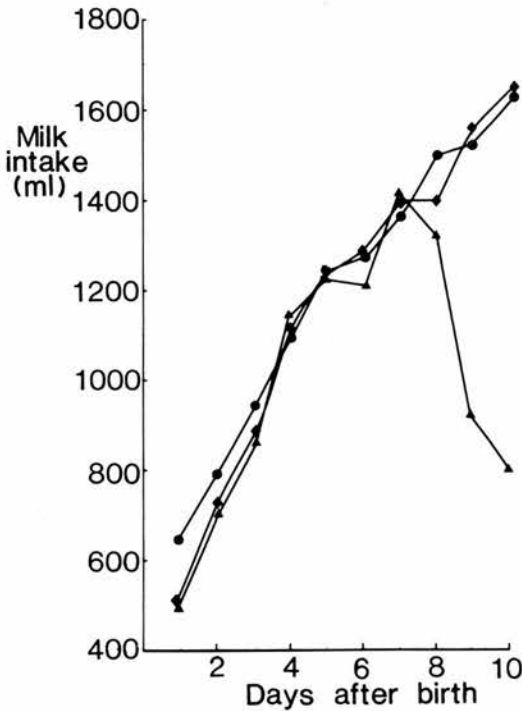


FIG. 1. Daily milk intake of lambs. Symbols: ●, lambs were inoculated with ETEC only at 5 days (lambs no. 3 and 4), rotavirus only at 8 days (lambs no. 15 and 16), and ETEC at 5 days and rotavirus at 4 days of age (lambs no. 5 and 6); ▲, lambs were inoculated with *Cryptosporidium* only at 6 days (lambs no. 17 and 18), *Cryptosporidium* at 6 days, and ETEC at 8 days (lambs no. 19 and 20), and *Cryptosporidium* at 6 days and rotavirus at 8 days of age (lambs no. 21 and 22); ◆, control (two lambs).

no. 9 and 10 developed slight depression, anorexia, and diarrhea with virus excretion, whereas lambs no. 11, 15, and 16 developed subclinical infections with rotavirus in their feces. Data were unavailable for lambs no. 9, 10, and 11, but no decrease in milk intake was observed for lambs no. 15 and 16 (Fig. 1).

Lambs no. 9 and 10 had lesions associated with rotavirus infection. The villi in the lower small intestine were stunted, and the lamina propria was infiltrated by mononuclear cells and eosinophils. Lambs no. 11 and 16 had no detectable morphological changes, but the distal intestine of lamb no. 15 contained patches of stunted villi which were, however, clothed by columnar cells. Infiltrates of mononuclear cells were seen in the lamina propria, but the crypts were not elongated. Specific IF studies for rotavirus showed extensive fluorescence in the epithelial cells of lambs no. 9 and 10, extending from the lower jejunum to mid-ileum. Very little specific

fluorescence was detected in gut sections obtained from lambs no. 11, 15, and 16.

(iii) *Cryptosporidium*. Four lambs were inoculated with homogenates containing *Cryptosporidium* oocysts. Two lambs, no. 12 and 13, infected at 1 day of age, developed profuse diarrhea, which soon became intermittent, and lamb no. 13 was moribund when it was killed at 6 days of age. Both lambs had markedly reduced milk intake. Lambs no. 17 and 18, infected when 6 days old, became extremely depressed and passed soft feces, and their milk consumption fell (Fig. 1). Lamb no. 17 died 2 days postinoculation.

Histological examination of the intestines of all four lambs revealed severe stunting and fusion of villi from the jejunum to the terminal ileum; the villi were covered with immature cuboidal cells. The lamina propria contained infiltrates of mononuclear cells. Both small and large intestines were heavily infected with the organism, but the large intestine was only slightly inflamed.

Mixed infections. (i) ETEC and rotavirus. Four lambs (no. 5, 6, 7, and 8) were inoculated with ETEC and rotavirus (Table 1). All four shed both microorganisms in their feces, but none had diarrhea, nor was there any decrease in milk intake (Fig. 1, lambs no. 5 and 6).

No pathological changes were seen in any intestinal site, and IF studies revealed no specific fluorescence against the ETEC, indicating that mucosal colonization had not occurred. However, some fluorescence was observed on the surface of the spiral colon and cecum in older lambs (no. 7 and 8). The extent and distribution of rotavirus-infected cells as detected by IF were similar to those for the single infections in lambs more than 2 days old, viz, patches of small groups of fluorescing cells.

(ii) *ETEC and Cryptosporidium*. Lambs no. 19 and 20 became extremely depressed within 24 h postinoculation with *Cryptosporidium*, and their milk intake was reduced (Fig. 1). ETEC were detected in the feces of these animals, but the bacterial infection did not aggravate the clinical response to *Cryptosporidium*.

Intestinal lesions in these two lambs were much more severe and extensive than in those infected with ETEC and rotavirus. Morphological changes, such as stunting, fusion, and bridging of villi, extended proximally as high as the upper jejunum, and replacement of enterocytes by immature cells was more extensive. Infiltration of the crypts by neutrophils from the lamina propria, with resultant crypt sepsis, was apparent. The large intestines were heavily infected with cryptosporidia. Specific IF tests for ETEC

revealed no mucosal colonization by the organism at any part of the intestine.

(iii) **Rotavirus and *Cryptosporidium*.** Lambs no. 21 and 22 were inoculated with *Cryptosporidium* at 6 days and rotavirus at 8 days of age, respectively (Table 1). Oocysts were present in the feces at 2 days postinoculation, but rotavirus was not detected in the feces at any time. The lambs were depressed, and their milk consumption dropped 2 days postinoculation with *Cryptosporidium* (Fig. 1).

The histological lesions associated with lambs no. 21 and 22 were similar to those observed for the lambs which have been inoculated with *Cryptosporidium* alone. IF studies for rotavirus revealed very few infected epithelial cells, as in the single infections with rotavirus at the comparable age.

Bacterial counts. Lamb no. 2, inoculated with ETEC, had high bacterial counts at every site of the small and large intestines (10^9 to 10^{10} viable organisms per 0.1 g of mucosal scraping) except the duodenum (10^7). All the other lambs, whether inoculated with ETEC alone or in combination with rotavirus or *Cryptosporidium*, had lower bacterial counts: 10^6 to 10^7 in the lower small intestine and 10^8 in the large intestine.

Enzymology. The lambs were grouped into subclinically affected (group 1), clinically affected (group 2), and controls (group 3) as follows. Group 1 consisted of lamb no. 14 (ETEC only), lambs no. 15 and 16 (rotavirus only), lambs no. 5 and 6 (ETEC and rotavirus). Group 2 consisted of lambs no. 17 and 18 (*Cryptosporidium* only), lambs no. 19 and 20 (ETEC and *Cryptosporidium*), and lambs no. 21 and 22 (rotavirus and *Cryptosporidium*). Group 3 consisted of five uninoculated age-matched controls.

Sites from the duodenum, lower jejunum, and lower ileum were examined for disaccharidase activity, and the mean enzyme level at each site for lactase, maltase, and sucrase was calculated. The lactase results are given in Table 2.

There were no significant differences between or within groups at any site for either maltase or sucrase activity. However, with lactase, signifi-

cant differences in activity were recorded between groups 1 and 2 ($P < 0.05$) in both the duodenum and lower ileum. In the latter site, a highly significant difference ($P < 0.01$) was obtained for the activities of groups 2 and 3.

DISCUSSION

The results of these experiments show that colostrum-deprived gnotobiotic lambs were susceptible to clinical infection with rotavirus or ETEC when less than 2 days of age, but subclinical infections were produced in lambs more than 4 days of age.

Lambs infected with *Cryptosporidium* all exhibited similar clinical responses, irrespective of whether they were separately challenged with rotavirus or ETEC. Clinical illness, reduced milk intake, reduced lactase activity in the small intestine, and mucosal damage correlated well in *Cryptosporidium*-infected lambs.

Clinical diarrhea and the pathogenesis of diarrhea in newborn lambs caused by ETEC (22) and lamb rotavirus (19) have been described. Subclinical rotavirus infections in lambs inoculated at up to 8 days of age were demonstrated by the shedding of virus in the feces and by IF of gut sections. The ETEC serotype used in this experiment possessed the K99 adherence antigen. However, inoculation of lambs with 10^{10} viable organisms failed to result in colonization of the small intestine of lambs aged 5 days or older, implying a close correlation between adherence, colonization, and clinical diarrhea, on the one hand, and the age of the lamb, on the other. The same organism (08:K87:K99) in combination with another strain (09:K30:K99) has been shown to induce severe diarrhea in three out of seven colostrum-fed young lambs (22), but the authors did not state at what age the lambs were infected.

Coinfection of 4- to 8-day-old lambs with ETEC and lamb rotavirus failed to induce clinical diarrhea, and the use of two SPF lambs did not alter the outcome. Neither evidence of bacterial adherence nor increased viral fluorescence resulted from the dual infection. No statistically significant difference in terms of disaccharidase activity among the control, ETEC-, rotavirus-, or rotavirus-plus-ETEC-infected lambs was observed. In contrast, coinfection of 2-week-old calves with rotavirus and ETEC induced diarrhea in circumstances where infection with a single agent would not (24).

The clinical manifestations, pathogenesis of diarrhea, and age susceptibility of newborn SPF lambs to *Cryptosporidium*, have been described elsewhere (Tzipori et al., in press). At that age *Cryptosporidium* infection induced severe diarrhea, but in lambs 6 days old intermittent diar-

TABLE 2. Lactase activity at different sites along the small intestines of infected and control lambs

| Groups ^a | No. of replicates | Lactase activity (mean \pm standard deviation) at: (μ mol/min per g of wet wt) | | |
|---------------------|-------------------|---|------------------------------|-----------------|
| | | Duodenum | Lower jejunum | Lower ileum |
| 1 | 4 | 6.69 \pm 1.61 | 8.00 \pm 2.03 | 0.93 \pm 0.42 |
| 2 | 5 | 2.11 \pm 0.60 ^b | 1.15 \pm 0.28 ^c | 0.28 \pm 0.15 |
| 3 | 5 | 4.65 \pm 0.49 | 4.33 \pm 0.79 | 1.85 \pm 1.52 |

^a See text for composition of groups.

^b $P < 0.05$ with respect to group 1.

^c $P < 0.05$ with respect to group 1. $P < 0.01$ with respect to group 3.

rhea and constipation were observed, indicating that continuous diarrhea is not a consistent clinical manifestation of enterocolitis in older SPF lambs. Terminal examination revealed that 18 h after feeding the stomach still contained large volumes of milk, and the small intestine, which lacked muscular tone and peristaltic action, often contained large amounts of fluid, while the large intestine contained hard and pelleted feces. Thus complete stasis of the gut apparently slowed the rate at which the stomach emptied, thus reducing the milk intake (Tzipori et al., in press).

From previous (Tzipori et al., in press) and the present experiments, it appears that *Cryptosporidium* in lambs is an enteric pathogen which induces a more severe disease in older lambs than does ETEC or rotavirus acting singly or in combination with each other.

Although the work reported here provides information regarding the possible outcome of mixed enteric infections in lambs, the findings do not parallel what happens in calves. Calves are susceptible to rotavirus infection for a longer period than lambs, and dual infection with rotavirus and ETEC caused diarrhea in sucking calves at least 2 weeks of age (24). Further, SPF or colostrum-fed calves older than 1 week appear to be more susceptible to experimental cryptosporidiosis than lambs of similar ages (Tzipori and Sherwood, unpublished data).

The choice of a 2-day interval between inoculations with *Cryptosporidium* (at 6 days) and ETEC or rotavirus (at 8 days) was an attempt to synchronize the incubation periods of the two microorganisms. Other time intervals may have different results. It is significant that the incubation period of *Cryptosporidium* seemed constant at 2 days as oocyst shedding in the feces was noted after this time. It could be argued, for instance, that infection with *Cryptosporidium* before rotavirus may have depleted the small intestine of mature enterocytes for rotavirus infection and replication (11) or, in the case of ETEC, receptor sites on the brush borders may have been blocked by an overwhelming cryptosporidial infection.

Although the *Cryptosporidium* inoculum was not quantitated, the same preparation was used throughout the experiment. Passage of the *Cryptosporidium* inoculum through SPF rats was considered to provide a "biological filtration" of gut bacteria. However, further work is needed to separate the oocysts from feces to obtain a more satisfactory source of infectious material.

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Passive Immunity in Calf Diarrhea: Vaccination with K99 Antigen of Enterotoxigenic *Escherichia coli* and Rotavirus

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Twenty-four pregnant cows were vaccinated intramuscularly with K99 extract from enterotoxigenic *Escherichia coli* and inactivated rotavirus as follows: six cows were injected with 2 ml of oil-adjuvanted vaccine; six cows were injected with 0.5 ml of oil-adjuvanted vaccine; six cows were injected with 4 ml of aluminum hydroxide-adjuvanted vaccine twice with a four-week interval; and six cows were unvaccinated as controls. Calves born to these cows were challenged with enterotoxigenic *E. coli* at 6 to 18 h after birth. Serum and milk antibodies to K99 and rotavirus in cows vaccinated with either dose of oil vaccine were significantly increased until at least 28 days after calving. In cows vaccinated with alhydrogel vaccine, there was a significant K99 antibody increase in serum and in colostrum but not in milk and a significant rotavirus antibody increase only in colostrum. Five of six calves born to unvaccinated cows developed enterotoxic colibacillosis after challenge, and all excreted the challenge strain of enterotoxigenic *E. coli*. None of the 18 calves in the three vaccinated groups developed clinical colibacillosis, and fecal excretion of the challenge organism was reduced. A combined enterotoxigenic *E. coli*-rotavirus vaccine may prove useful in preventing some outbreaks of calf diarrhea.

Although many infectious agents have been implicated in the etiology of diarrhea in young calves, four microorganisms stand out as being of widespread occurrence and proven enteropathogenicity: rotavirus, coronavirus, enterotoxigenic *Escherichia coli* (ETEC), and cryptosporidia (2, 8, 10, 11, 23).

Control of diarrhea has been attempted with a live attenuated rotavirus-coronavirus vaccine for oral inoculation of newborn calves (Scourvax-II; Norden Laboratories) but has not been proven effective in blind field trials (3, 9). Control can also be attempted through dam vaccination to elevate the titers of specific antibody ingested by the calf in colostrum and milk. Such an approach has been used successfully with both bacterins and with K99 pili from ETEC (1, 14, 15), although live attenuated rotavirus-coronavirus vaccination of pregnant cows (Calf Guard; Norden Laboratories) does not significantly raise milk antibody titers (L. L. Myers and D. R. Snodgrass, J. Am. Vet. Med. Assoc., in press). The use of inactivated adjuvanted rotavirus vaccine results in greatly increased colostrum and milk antibody production (20, 21).

The objectives of this study were to combine K99 pili from ETEC with a rotavirus vaccine for pregnant cows and to assess the efficacy by

serology and by challenging newborn calves with ETEC.

MATERIALS AND METHODS

Animals. A total of 24 pregnant hill suckler cows from 7 to 15 years of age were allocated to treatment groups at 4 to 10 weeks before calving. Six cows were vaccinated once with 2 ml of oil-adjuvanted vaccine by deep intramuscular injection in the neck; six cows were similarly vaccinated once with 0.5 ml of the same oil-adjuvanted vaccine; six cows were vaccinated twice with a 4-week interval with 4 ml of aluminum hydroxide-adjuvanted vaccine by deep intramuscular injection in the neck; and six cows were not vaccinated. Four of the cows allocated to the control group had been vaccinated with rotavirus-coronavirus vaccine in their previous pregnancy and were included as controls only for the ETEC component.

The cows were housed before calving for the duration of the experiment. Calves were challenged with ETEC between 6 and 18 h after birth. After challenge, cow-calf pairs from the different treatment groups were kept apart for at least 3 days to prevent cross-suckling and then were moved to a pen separate from that of the uncalved cows.

***E. coli* cultures.** The following serotypes of *E. coli* were used: O101:K:K99 (designated ETEC 1); O9:K30(B):K99 (designated ETEC 2); O9:K35(A):K99 (designated ETEC 3); and O8:K85ab:K99 (designated ETEC 4).

Vaccines. For the preparation of alhydrogel-adjuvanted vaccine, the K99 component was derived from

TABLE 1. Mean K99 antibody titers (measured by ELISA) in cow serum, cow whey, and calf serum after K99-rotavirus vaccination

| Vaccine ^a | Titer (log ₁₀) in cow serum | | | Titer (log ₁₀) in whey at following day after calving: | | | | | | Titer (log ₁₀) in calf serum at following day of age | |
|----------------------|---|------------------------|------------------|--|------------------|------------------|------------------|------------------|------------------|--|------------------|
| | Before vaccination | 4 Wk after vaccination | At calving | 1 | 3 | 7 | 14 | 28 | 90 | 3 | 28 |
| None (control) | 1.9 | 1.8 | 1.8 | 2.0 | 1.7 | 1.7 | 1.7 | 1.7 | 1.7 | 1.8 | 3.2 |
| Oil (2 ml) | 1.5 | 4.2 ^b | 4.5 ^b | 4.3 ^b | 4.1 ^b | 3.6 ^b | 3.5 ^b | 3.1 ^b | 2.9 ^b | 4.3 ^b | 4.0 ^b |
| Oil (0.5 ml) | 2.0 | 3.9 ^b | 4.0 ^b | 4.3 ^c | 3.9 ^b | 3.3 ^b | 2.8 ^b | 2.3 ^c | 2.6 ^b | 4.2 ^b | 3.6 |
| Alhydrogel | 1.7 | 2.1 | 3.1 ^b | 3.1 ^b | 2.6 ^b | 1.9 | 1.9 | 1.7 | 1.7 | 2.3 ^c | 2.6 |

^a There were six cows in each vaccine group.^b Differs significantly from control ($P < 0.01$).^c Differs significantly from control ($P < 0.05$).

the culture supernatant of ETEC 1, which was grown in a synthetic medium containing selected amino acids, trace salts, and lactose and was buffered with phosphates to pH 7.5. After 8 to 10 h of incubation at 37°C in an aerated vessel, the culture was inactivated in situ at 60°C for 30 min. The cells were separated aseptically from the supernatant by centrifugation. After measurement of K99 antigen in the sterile culture supernatant, 20% alhydrogel was added.

Tissue culture-adapted calf rotavirus was prepared and inactivated as previously described (20). The rotavirus and K99 components were blended aseptically with alhydrogel in the proportions 1:1:8 so that each milliliter of the combined vaccine contained 15 U of K99 and $10^{5.4}$ 50% tissue culture infective doses of rotavirus before inactivation.

For the preparation of oil-adjuvanted vaccine, the K99 component was derived from ETEC 1 grown on 5% horse blood agar at 37°C for 18 h. The growth was harvested in sterile saline and concentrated by centrifugation so that it contained 2.50×10^{11} cells per ml. Portions (20 ml each) of the concentrate were homogenized in a Silverson homogenizer for 4 min at 0°C. The cells were separated from the supernatant at $20,000 \times g$ for 30 min; then 0.1% Merthiolate was added, and the preparation was heated at 60°C for 30 min. The sterility of this crude K99 extract was checked, and its K99 content was measured before use.

Rotavirus and K99 were mixed with 0.2% Tween 80, and this aqueous phase was emulsified with 2 volumes of oil adjuvant (90% Marcol 52 [Esso], 10% Arlacel A [Sandria Chemicals]). Each milliliter of vaccine contained 60 U of K99 and $10^{5.2}$ 50% tissue culture infective doses of rotavirus before inactivation.

ETEC challenge. Strain B44 (ETEC 2) was grown in Trypticase soy broth (BBL Microbiology Systems) for 8 h and then on Minca-IsoVitaleX (BBL) (6) agar for 18 h at 37°C. The bacteria were suspended in phosphate-buffered saline with 10% dimethyl sulfoxide and stored in 10-ml aliquots at -70°C.

To inoculate each calf, one 10-ml aliquot was thawed and given orally by syringe. The mean inoculum titer was 4.1×10^{10} colony-forming units per 10 ml (range, 3.0×10^{10} to 6.8×10^{10} colony-forming units per 10 ml). No decrease in the inoculum titer occurred over the 2-month experimental period. The enteropathogenicity of the stored organisms was confirmed periodically by slide agglutination for K99 and the

infant mouse test for heat-stable toxin production (4).

K99 serology. Antibodies to K99 were assayed by enzyme-linked immunosorbent assay (ELISA) or passive hemagglutination (PHA). The ELISA utilized rabbit anti-K99 immunoglobulin G (kindly supplied by W. H. Jansen) as capture antibody, followed successively by K99 antigen, test serum or whey, and rabbit anti-bovine immunoglobulin G (Miles Laboratories, Inc.) conjugated with alkaline phosphatase. The final phosphatase substrate (Sigma Chemical Co.) reaction was read at 405 nm after 2 h at room temperature. In each test, doubling dilutions of a standard bovine anti-K99 serum were included. The titers of the test samples are expressed in relation to a calibration curve calculated from the standards.

In the PHA assay, pyruvic acid-stabilized sheep erythrocytes (7) were sensitized with K99 antigen from ETEC 4. A suspension of erythrocytes in 0.1 M acetate buffer (pH 4.5) was coated to saturation with K99 derived by the method of Morris et al. (12), washed five times in phosphate-buffered saline (pH 7.5), and resuspended to 1% (vol/vol). Test sera and whey samples were adsorbed with an equal volume of packed unsensitized erythrocytes for 18 h at 4°C to remove nonspecific hemagglutinins. Serial doubling dilutions of serum or whey samples in 0.3% Formol saline were prepared in microtiter plates, and an equal volume (0.025 ml) of sensitized erythrocytes was added. The agglutination pattern was read after 18 h of incubation at 37°C. All of the samples were tested on one occasion, although repeat tests on selected samples yielded the same titers.

Rotavirus serology. Serum samples, whey from colostrum samples, and whey from milk samples were tested for the presence of neutralizing antibody to tissue culture-adapted calf rotavirus on bovine embryo kidney cells or MA104 cells grown in microtiter plates.

Titration of K99 antigen. The titration method used is based on the capacity of the K99 antigen to adsorb K99 antibodies from a standard antiserum of known titer, which is then titrated for residual antibody by PHA. This method is based on an in vitro assay designed for quantitating K88 antigens of *E. coli* (16). The results are expressed as agglutinin absorbing units. The standard antiserum used was produced in a pig vaccinated with a sterile culture supernatant of ETEC 3. The serum was adsorbed to remove all detectable O9 and K35(A) agglutinins.

Toxicity of K99 components in combined vaccines. The toxicity of the K99 vaccine preparations was examined by injecting groups of 10 mice intraperitoneally with 15 U of alhydrogel-adsorbed K99 and 180 U of crude K99 extract.

Feces examination. Calf feces samples were examined for rotavirus by ELISA (5) and for cryptosporidia by examination of Giemsa-stained fecal smears (19). Samples were cultured on Minca-IsoVitalX (6) and MacConkey agars overnight aerobically at 37°C. Five colonies grown on Minca-IsoVitalX agar were tested for K99 by slide agglutination with rabbit antiserum to strain K12:K99 adsorbed with the K12 strain.

At intervals, fecal swabs from all calves were tested for the presence of *Salmonella* spp. after overnight enrichment in Selenite broth and for *Campylobacter* spp. by growth on 5% sheep blood agar plus Skirrows antibiotic supplement (Oxoid Ltd.) under microaerophilic conditions at 37°C.

Observations. Each cow was bled for serum at vaccination, 4 weeks after vaccination, at parturition, and 28 days after parturition. Colostrum and milk samples were collected at 1, 3, 7, 14, 28, and 90 days after calving. The calves were bled for serum at 3 and 28 days of age. Feces samples were collected from the calves daily for 6 days. A sample of these feces was taken for microbiological examination, and the remainder was dried to constant weight for dry-matter estimation. The calves were weighed at 1, 2, and 3 days of age. All calves were examined clinically at least once a day for 6 days and assigned a clinical score on a subjective scale similar to that used by Myers (14): (i) normal, feces firm; (ii) transient diarrhea within 24 h of inoculation, lasting only a few hours; (iii) severe watery diarrhea, calf becoming dehydrated and dull; (iv) severe watery diarrhea, calf too weak to stand, with death ensuing.

RESULTS

K99 immunological response measured by ELISA. Six cows had low preexisting serum antibody (mean titer, 158), whereas all other cows were negative (titer < 100). All vaccine regimes significantly increased serum antibody titers ($P < 0.001$) (Table 1), although oil vaccines produced significantly higher titers at calving (18,900 and 8,830 for 2- and 0.5-ml doses, respectively) than did the alhydrogel vaccine (1,190) ($P < 0.001$).

Both 2- and 0.5-ml doses of oil vaccine produced very high titers in colostrum whey (32,100 and 19,800, respectively) compared with controls (108) ($P < 0.001$). These colostrum antibody levels declined slowly throughout the 28-day observation period, but remained significantly higher than in the controls (2 ml of oil, $P < 0.001$; 0.5 ml of oil, $P < 0.05$). Antibody titers in colostrum whey from the cows vaccinated with alhydrogel vaccine were also increased (1,140, $P < 0.001$), but by 7 days after calving, antibody was no longer detectable in this group.

The serum antibody titers in 3-day-old calves reflected the colostrum antibody titers of their dams. By 28 days of age, the calves born to

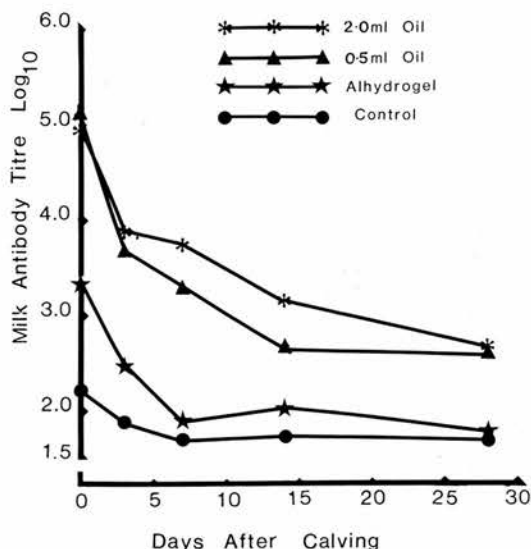


FIG. 1. K99 antibody titers determined by PHA (\log_{10}) in colostrum and milk of cows during the first 28 days of lactation.

unvaccinated cows had developed high serum antibody titers to K99 as a result of ETEC infection.

K99 immunological response measured by PHA. The assay of sera and wheys by PHA confirmed the results obtained by ELISA (Fig. 1). In addition, the cow sera collected 28 days after calving were tested, and K99 antibody titers were found to be still significantly raised in all vaccinated cows.

Rotavirus immunological response. The results from the four control cows which had been vaccinated in a previous pregnancy are excluded from these results, and data from seven extra control cows from the same farm not otherwise included in the experiment are incorporated. All cows had prevaccination serum antibody to rotavirus. The serum and milk antibody responses of cows to both doses of oil-adjuvanted vaccine were significant (Table 2).

Cows vaccinated with alhydrogel-adjuvanted vaccine had raised antibody titers in serum and milk, but only colostrum antibody titers were significantly higher than in control cows.

Response of calves to ETEC challenge. After ETEC challenge, five of six calves from control cows developed acute enteric disease (disease rating iii or iv) characterized by profuse watery diarrhea, dehydration, and dullness (Table 3). The mean body weight loss of 5.7% and the mean minimum fecal dry matter of 8.4% confirmed the severity of the disease. One calf died 2 days after challenge.

The clinical responses of calves in all three

TABLE 2. Mean rotavirus antibody titers (measured by the neutralization test) in cow serum and whey after K99-rotavirus vaccination

| Vaccine | Titer (log ₁₀) in cow serum | | | Titer (log ₁₀) in whey at following day after calving: | | | | |
|----------------|---|------------------------|-------------------|--|-------------------|-------------------|------|-------------------|
| | Before vaccination | 4 Wk after vaccination | At calving | 1 | 3 | 7 | 14 | 28 |
| None (control) | 2.51 | 2.66 | 2.36 | 2.81 | 2.05 | 2.02 | 1.83 | 1.70 |
| Oil (2 ml) | 2.66 | 3.19 | 3.46 ^b | 4.06 ^a | 3.51 ^b | 2.86 ^a | 2.51 | 2.26 ^a |
| Oil (0.5 ml) | 2.81 | 3.71 ^a | 3.65 ^b | 3.96 ^a | 3.46 ^b | 2.91 ^a | 2.41 | 2.43 ^a |
| Alhydrogel | 2.66 | 3.33 | 3.29 | 3.51 ^b | 2.81 | 2.36 | 2.15 | 2.02 |

^a Differs significantly from control value ($P < 0.01$).^b Differs significantly from control value ($P < 0.05$).

vaccinated groups were similar. A mild transient diarrhea unaccompanied by systemic disturbance was observed in half of the calves (disease rating ii). All calves gained weight, and fecal dry matter did not fall below 16% in any individual.

Microbiological examination of feces. A serological examination of fecal coliforms was performed for 6 days after ETEC challenge. In control calves, 86% of *E. coli* could be identified as ETEC (Table 4). The proportion of B44 excreted in the feces of calves from vaccinated cows was reduced. In only one calf was there no detectable B44 excretion. Rotavirus was detected in the feces of one control calf with diarrhea and in three clinically normal calves born to vaccinated cows. Cryptosporidial oocysts were not observed in the feces of any calf.

Vaccine toxicity. No toxic effects of K99, used in the vaccines, were detected in mice, and all vaccinated cows remained clinically normal.

DISCUSSION

Calves sucking dams vaccinated with K99 pili in any of the schedules used were protected against the clinical effects of challenge with ETEC, whereas the same challenge produced severe enterotoxic colibacillosis in calves from control cows. In addition, excretion of the challenge strain was significantly reduced in calves from vaccinated cows. Although the oil vaccines

produced a much higher immunological response than the alhydrogel vaccine, the degree of protection under these experimental conditions was equally satisfactory.

Most natural ETEC infections occur in calves 1 to 2 days old (2, 10), and increased antibody titers in colostrum alone should be protective in these cases. However, there is experimental evidence that initial rotavirus infection can facilitate ETEC colonization in calves up to 1 week old (17, 20a), and such dual infection of older calves has been reported to occur naturally (10). For this reason, it is desirable to stimulate production of K99 antibody in milk as well as in colostrum, and the oil-adjuvanted vaccine formulation was more effective in this regard than the alhydrogel vaccine. The mean K99 ELISA titers of 7-day milk of cows vaccinated with either dose of the oil-adjuvanted vaccine were higher than those in colostrum of cows which received the alhydrogel-adjuvanted vaccine. As calves of the latter group of cows were resistant to experimental challenge, it is reasonable to suggest that cows vaccinated with oil-based K99 vaccine would confer protection to their calves for at least 7 days and probably longer.

With the methods of vaccine production used with strain B41 (O101:K-) (K99) it is likely that antigens other than K99 were present, in particular the O101 somatic antigen and an anionic adhesin (13). However, cross-protection against

TABLE 3. Clinical results of ETEC challenge

| Vaccine | No. of calves given the following disease rating: ^a | | | | Wt at 48 h/wt at birth (%) ^b | Minimum fecal dry matter (%) ^{b,c} |
|----------------|--|----|-----|----|---|---|
| | i | ii | iii | iv | | |
| None (control) | 0 | 1 | 4 | 1 | 94.3 ± 3.6 | 8.4 ± 2.1 |
| Oil (2 ml) | 3 | 3 | 0 | 0 | 107.5 ± 1.5 ^d | 28.7 ± 3.2 ^d |
| Oil (0.5 ml) | 3 | 3 | 0 | 0 | 106.7 ± 1.6 ^d | 24.9 ± 2.9 ^d |
| Alhydrogel | 3 | 3 | 0 | 0 | 103.3 ± 1.5 ^e | 22.6 ± 1.8 ^d |

^a See text for definitions of disease ratings.^b Mean ± standard error.^c Minimum recorded for each calf over the 4 days after ETEC challenge.^d Differs significantly from control ($P < 0.01$).^e Differs significantly from control ($P < 0.05$).

TABLE 4. Fecal excretion of ETEC after challenge

| Vaccine | Colonies agglutinated (%) ^a |
|----------------|--|
| None (control) | 86 |
| Oil (2 ml) | 49 ^b |
| Oil (0.5 ml) | 16 ^c |
| Alhydrogel | 32 ^c |

^a Mean number of colonies agglutinated by K99 antiserum as a percentage of the total number of colonies tested over the 6 days after challenge.

^b Differs significantly from control ($P < 0.05$).

^c Differs significantly from control ($P < 0.01$).

the challenge strain O9:K30:K99 suggests that effective protection was produced in this case by K99 antibodies, even against a strain which may possess colonization properties through K30 (18).

These results confirm those of others who have found that antibody to K99 protects against virulent ETEC challenge (1, 15). However, the incorporation of a rotavirus vaccine greatly increases the value of the immunization regime. No rotavirus challenge was included, but the rotavirus serological response produced in this experiment by the oil-adjuvanted vaccines was consistent with that produced previously, which has been shown to confer substantial protection against rotavirus infection (5, 20, 21). Thus, there was no evidence that the inclusion of K99 interfered with the maternal response to rotavirus vaccination.

An effective bivalent vaccine against ETEC and rotavirus would not prevent all calf diarrhea outbreaks. In particular, disease due to calf coronavirus would continue, as has been found with a previous rotavirus vaccine (22), and cryptosporidiosis would continue to be present. However, the use of such a vaccine could be expected to lead to a useful reduction in morbidity and mortality from diarrhea in young suckled calves and potentially in dairy calves also if the duration of colostrum feeding was prolonged (21).

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A FIELD SURVEY OF THE AETIOLOGY OF NEONATAL CALF DIARRHOEA

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INTRODUCTION

The aetiology of neonatal calf diarrhoea remains obscure. Surveys of field outbreaks of diarrhoea are few (5, 6) and only recently have sufficient knowledge and techniques been available to allow reasonably comprehensive investigations of the potential bacterial, viral, and protozoal aetiologies to be made. This paper describes an attempt at such a survey in dairy and beef herds in Scotland and the north of England. The survey is continuing, and results from 1981 and the first 3 months of 1982 are presented.

MATERIALS AND METHODS

As each outbreak of diarrhoea was brought to our attention, faeces samples were collected from freshly-affected calves at the onset of diarrhoea before treatment was given, and from normal calves of similar age where possible. A minimum of four diarrhoeic calves was sampled on each farm included in the survey.

Bacteriological examination was carried out on the faeces for *Salmonella*, enterotoxigenic *Escherichia coli* (ETEC) (4) and *Campylobacter* (2). Faeces were examined by electron microscopy for enteric viruses, by enzyme-linked immunosorbent assay for rotavirus and coronavirus (3), and by haemadsorption elution haemagglutination assay for coronavirus (1). Giemsa-stained faecal smears were examined for cryptosporidia.

RESULTS

Occurrence of enteropathogens in diarrhoeic calves.

Six dairy (Table 1) and fourteen beef suckler (Table 2) herds have been examined.

TABLE 1.

Detection of enteropathogens in diarrhoeic calves in 6 dairy herds.

| Farm | Enteropathogens detected/no. of samples examined. | | | | |
|------|---|-------------|-----------------|------|---------------|
| | rotavirus | coronavirus | cryptosporidium | ETEC | campylobacter |
| 1. + | 40/43 | 2/28 | 1/37 | 0/43 | N.T. |
| 2. | 10/17 | 1/17 | 2/17 | 0/17 | 0/12 |
| 3. | 1/4 | 1/4 | 1/4 | 0/4 | N.T. |
| 4. | 4/8 | 0/4 | 1/8 | 0/4 | N.T. |
| 5. | 4/5 | 1/5 | 0/5 | 0/5 | N.T. |
| 6. | 5/13 | 4/13 | 6/13 | 0/13 | N.T. |

+ *Salmonella* sp. isolated from one calf. N.T. not tested.

TABLE 2

Detection of enteropathogens in diarrhoeic calves in 19 beef suckler herds.

| Farm | Enteropathogens detected/no. of samples examined | | | | |
|-----------------|--|-------------|-----------------|------|---------------|
| | rotavirus | coronavirus | cryptosporidium | ETEC | campylobacter |
| 7. | 0/4 | 0/4 | 1/4 | 0/4 | N.T. |
| 8. | 3/8 | 0/8 | 1/8 | 0/4 | 6/8 |
| 9. | 0/13 | 0/13 | 6/13 | 0/13 | N.T. |
| 10. | 0/5 | 1/5 | 0/5 | 0/5 | N.T. |
| 11. | 4/7 | 5/7 | 0/7 | 0/7 | N.T. |
| 12. | 0/4 | 0/4 | 0/4 | 4/4 | N.T. |
| 13. | 5/7 | 0/7 | 0/7 | 0/7 | N.T. |
| 14 ⁺ | 4/7 | 0/7 | 2/7 | 0/7 | N.T. |
| 15. | 3/4 | 0/4 | 1/4 | 0/4 | N.T. |
| 16. | 0/4 | 0/4 | 1/4 | 2/4 | N.T. |
| 17. | 5/5 | 2/5 | 1/5 | 0/5 | N.T. |
| 18. | 4/8 | 1/8 | 1/8 | 0/8 | N.T. |
| 19. | 1/9 | 2/9 | 2/9 | 0/9 | N.T. |
| 20. | 13/24 | 1/24 | 13/24 | 0/24 | 10/24 |

+ Salmonella sp. isolated from one calf. N.T. not tested

Comparison of Diarrhoeic and Normal Calves

Samples were obtained also from normal calves of similar age on 10 of the affected farms (Table 3).

TABLE 3

Comparison of enteropathogen excretion rates in diarrhoeic and normal calves on 10 farms

| Enteropathogen | Diarrhoeic calves | Normal calves | Significance (p) |
|---------------------------------|-------------------|---------------|------------------|
| rotavirus | 40/90 | 5/36 | < 0.01 |
| coronavirus | 13/90 | 2/36 | N.S. |
| cryptosporidium | 25/90 | 6/36 | N.S. |
| ETEC | 6/90 | 0/36 | N.S. |
| campylobacter (3 farms only) | 16/44 | 5/15 | N.S. |

analysis by χ^2 test. N.S. not significant

These results show a significant association with diarrhoea only for rotavirus. However, it is possible that other less common enteropathogens were associated with diarrhoea on a few farms, but that this significance was submerged in a greater number of casual occurrences on other farms. To examine this possibility, the prevalence of each enteropathogen was recalculated only for the farms on which more than one third of diarrhoeic calves excreted that particular enteropathogen (Table 4).

TABLE 4

Comparison of enteropathogen excretion rates in diarrhoeic and normal calves on farms where more than a third of diarrhoeic calves excreted the enteropathogen concerned.

| Enteropathogen | No. of farms | Diarrhoeic calves | Normal calves | Significance (p) |
|-----------------|--------------|-------------------|---------------|------------------|
| rotavirus | 7 | 45/81 | 3/27 | < 0.001 |
| coronavirus | 2 | 9/20 | 0/8 | 0.06 |
| cryptosporidium | 2 | 19/37 | 0/8 | 0.02 |
| ETEC | 2 | 6/8 | 0/5 | 0.02 |
| campylobacter | 2 | 16/32 | 3/9 | N.S. |

This analysis demonstrates that, in addition to rotavirus, cryptosporidium and ETEC were also significantly associated with diarrhoea on particular farms and the correlation of coronavirus excretion and diarrhoea approached significance. Campylobacter sp., on the other hand, were excreted equally frequently in diarrhoeic and normal calves, and their enteropathogenicity remains unproved.

Diagnosis

As these infectious agents are significantly associated with diarrhoea, it may be possible to reach a diagnosis from the 20 farm surveys presented in Tables 1 and 2, using the same arbitrary standard that an enteropathogen may be considered significant if excreted by more than one third of diarrhoeic calves (Table 5).

TABLE 5

Suggested aetiologic diagnosis on the survey farms

| Diagnosis | No. of farms |
|--|--------------|
| rotavirus | 9 |
| ETEC | 2 |
| cryptosporidium | 1 |
| rotavirus + coronavirus | 2 |
| rotavirus + cryptosporidium | 1 |
| rotavirus + coronavirus + cryptosporidia | 1 |
| unknown | 4 |

Age affected

Calves on 16 of the 20 farms were initially affected at 3-21 days of age, and this included farms under all the diagnoses listed in Table 5 except ETEC. The age of diarrhoeic calves on farms 12 and 16 where ETEC was diagnosed was mainly in the range 1-3 days, although occasional calves up to 6 days of age were affected. This confirms previous suggestions that ETEC affects mainly calves under 3 days old. Although rotavirus normally affects calves older than 3 days, on farm 5 calves suffered from rotavirus diarrhoea by 1 day of age indicating that under conditions of particularly bad hygiene rotavirus can infect younger calves.

DISCUSSION

The results to date of this ongoing survey suggest that it is possible to detect one or more enteropathogens at high prevalence rates in most calf diarrhoea outbreaks. The most commonly encountered organism which was significantly associated with diarrhoea was rotavirus, and of lesser frequency but still of significance were ETEC, coronavirus and cryptosporidium. *Campylobacter* sp. were relatively common but showed no apparent association with diarrhoea. *Salmonella* sp. were isolated from single calves on only 2 farms. However, if this survey had included farms rearing calves bought in from markets, it is reasonable to suggest that salmonellae may have been found more frequently.

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SUMMARY

Faeces samples were collected from untreated diarrhoeic and apparently normal calves on 20 farms, and examined for bacterial, viral, and protozoal enteropathogens. Rotavirus, enterotoxigenic *Escherichia coli* (ETEC) and cryptosporidium were all significantly associated with diarrhoea; the association of coronavirus and diarrhoea approached significance; and *Campylobacter* sp. occurred equally commonly in diarrhoeic and normal calves. The final diagnosis was rotavirus (9 farms); ETEC (2 farms); cryptosporidium (1 farm); rotavirus and coronavirus (2 farms); rotavirus and cryptosporidium (1 farm); rotavirus and coronavirus and cryptosporidium (1 farm); unknown (4 farms).